Effects of Neonatal Testosterone on Intermittent Access Ethanol Consumption in Female C57BL/6J Mice.

Alexandra M. Geada, Lara S. Hwa, Joseph F. DeBold, Klaus A. Miczek

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Abstract

Sex differences in alcohol consumption are known to occur in human and mouse models. Female C57BL/6J mice consume more alcohol during intermittent access schedules than males, a difference that may be influenced by gonadal hormones. To evaluate the influence of organizational effects of these hormones, a neonatal injection of testosterone was administered within 24 hours of birth. Females that were given these hormonal injections were found to drink less than control females injected with the sesame oil, the vehicle. This indicates a possible organizational effect of testosterone on alcohol drinking patterns. The interaction of alcohol and ovarian hormones was also examined by comparing estrous cyclicity in alcohol drinking and water drinking female C57BL/6J mice. There was no difference in alcohol consumption between stages of the estrous cycle. However, ethanol drinking was shown to alter normal cyclicity, with females spending more time in the diestrus stage compared to proestrus and estrus stages. Future investigations into the organizational effects of testosterone using neonatal castration or androgen blockers could yield more insight into this phenomenon.

Introduction

Alcoholism and Society

According to the National Institutes of Health, 18 million people in the United States suffer from an alcohol use disorder (NIAAA). Alcoholism is a serious public health problem and subtracts an average of 4.2 disability adjusted life years (DALYs) per person (Merikangas & Risch, 2003). Alcohol consumption is responsible for 2.5 million deaths worldwide (WHO, 2011). Apart from being a chronic relapsing disorder, it affects not only the people suffering from the disease but also those around them.

Progression from dependence to abuse, as well as drug sensitivity and vulnerability to the reinforcing effects of alcohol, differ between males and females (Lynch, Roth & Carroll 2002). In terms of alcohol consumption, heavy episodic drinking is more common in adult men, 17.9%, compared to women, 4.5% (Hibell et al. 2009; WHO 2011). Men also have higher rates of alcohol dependence and abuse compared to women (Brady & Randall 1999). While men are traditionally more likely to suffer from alcohol related disorders, the gender gap has decreased from 22.6% in 1975 to 12.3% in 2001 (Wallace et al. 2003). The increasing rate of women suffering from this disease has shifted the focus of current research to examine the underlying factors in this disparity (Hasin et al. 2007). Research examining the underlying causes of the gender differences in alcoholism can prove helpful in understanding the epidemiology of this disease.

Prior to puberty, the rates of alcohol consumption are more similar and in some cases females drink more than males (Biehl et al. 2007). Rates of underage current, binge, and heavy drinking for males and females ages 12-20 are very similar (NSDUH 2011). The fact that the sex differences emerge during this period of maturation suggests that biological mechanisms,

specifically gonadal hormones, influence this phenomenon (Witt 2007). Insight into the relationship between alcohol and hormones can lead to advances in understanding and preventing this disease.

Gender Differences in Alcohol Consumption and Dependence

Other disorders such as depression have gender differences that emerge during puberty (Angold & Worthman 1993), suggesting a hormonal influence. Puberty is the process of physical changes that allow for sexual reproduction. While there is variance among countries, females tend to go through puberty about 1-2 years earlier than males (Herman-Giddens et al. 2012). Girls begin puberty around the ages of 10-11 and finish around 15-17 years old. Boys begin around ages 12-13 and finish around 16-18 years old. Changes around this age include growth spurts, skeletal changes, increase in muscle and fat tissue (Nussey & Whitehead 2001), all of which are mediated by gonadal hormones. During this time, sexual organs begin releasing hormones, specifically androgens and estrogens, which allow for sexual maturation and sexual reproduction. In females, ovaries produce estrogens including estradiol. In males, the testes produce androgens, mainly testosterone.

Puberty has shown to influence drinking patterns in males and females. Between the ages of 12-17, the rates of drinking are the same between genders (13.3% and 13.3%)(NSDUH, 2011). After age 17, though, the gender differences widen and remain disparate with women drinking less alcohol, and experiencing less dependence and abuse than males (Witt 2007). In fact, early puberty is correlated with increased alcohol use independent of age and school grade (Patton et al. 2004; de Water et al. 2013; Biehl et al. 2007). All of these facts indicate that the gonadal hormones that emerge during puberty influence alcohol-drinking patterns.

While men are known to consume more alcohol than women, women experience higher blood alcohol concentrations (BACs) with lower alcohol intake than males (White et al. 2010; Nolen-Hoeksema 2004).

Additionally, women experience increased alcohol-related physical illnesses at lower levels of alcohol consumption as well as increased negative consequences of heavy alcohol consumption and alcohol use disorders (Nolen-Hoeksema 2004). This discrepancy may be due to the difference in weight of males and females, with females typically weighing less than males. Additionally, females have lower water content than males, and thus, have a smaller volume of ethanol distribution (Frezza et al. 1990). Women also are more likely to undergo a process called "telescoping" which refers to a more rapid progression from causal alcohol consumption to compulsive, habitual drinking behaviors (Lynch, Roth & Carroll 2002). While men are known to drink more than females, these findings indicate a need for research in understanding why women are more vulnerable to the negative effects of alcohol.

Drinking patterns in humans are under many other influences, including social, cultural, as well as biological. The reason women drink less alcohol than men may be due to the greater social sanctions against women drinking (Gomberg 1988). Similarly, alcohol consumption may be seen as part of the male gender role and discouraged as part of the female gender role (Chassin, Tetzloff, & Hershey 1985). The complexity of these social factors may be confounding the true influence of hormones. Animal models can be used to examine the influence of hormones exclusively, without the conflicting influences of social and cultural norms.

Sex differences in Preclinical Alcohol Research

Animal models of alcohol abuse and dependence have been critical in the understanding of alcohol related disorders as well as prevention and treatment for humans (Lynch, Roth &

Carroll 2002). While patterns of alcohol consumption differ between species, non-human primates exhibit drinking patterns similar to that of humans, with males drinking more than females, as well as constituting a higher percentage of heavy drinking (Vivian et al. 2001; Grant & Johanson 1988). The opposite phenomenon is seen in other species, though. Studies in rats, mice, and vervet monkeys show that females consume more alcohol than males, (Lancaster & Spiegel 1992; Juarez et al. 1993; Juarez & Barrios de Tomasi 1998). In rodent models, females drink more than males when having continuous as well as limited access to ethanol (Levinson et al. 2011; Middaugh et al. 1999). Additionally, females exhibit an increased preference for high concentrations of alcohol, which could be due to higher rates of ethanol metabolism and development of metabolic tolerance (Middaugh et al. 1999).

While the trend in rodents is opposite to that of humans, the onset of these drinking patterns emerges during puberty, indicating that similar hormonal influences are mediating these changes. Using rodent models can thus allow insight into the effects of hormones on drinking patterns in humans. Along with alcohol consumption patterns, differences in withdrawal symptoms between sexes can also provide insightful information.

Biological Sex Differences

Biological differences between males and female mammals are mediated by gonadal hormones as well as to a lesser extent, sex chromosomes. The X and Y-chromosomes direct the differentiation of the gonads into testis or ovaries (Haseltine & Ohno 1981). Males have one X and one Y chromosome and females have two X chromosomes. Genes encoded in the Y chromosome are expressed only in the brains of males, while genes on the X chromosome may be expressed at a different level between males and females (Becker et al. 2005). These chromosomes mediate the development of the gonads. The gonads secrete hormones, which are

responsible for the sexual differentiation between males and females. Along with the nervous system, the endocrine system is the primary regulatory system for the entire body (Emanuele & Emanuele 1997).

The hormones secreted by the gonads can be activational or organizational, depending on the time of secretion (Sisk & Zehr 2005). The activational effects of these hormones facilitate behavior in specific social contexts and are transient. These effects are typically seen in adulthood. Organizational effects refer to the structural sculpting of the nervous system, which occurs during development, especially during prenatal and early neonatal development (Phoenix et al. 1959), but also later in life. These effects are permanent and persist beyond the period of secretion of the hormones. The organizational effects of hormones on animal development have been seen in animals such as chimpanzees, rodents, and fish (Reinisch 1974).

The presence of testosterone during this critical period of central nervous system development influences the expression of behaviors and has a masculinization effect (Reinisch 1974). Testosterone is the main male hormone that is secreted during late gestational and neonatal periods as well as during adulthood. It is responsible for male development; removal of the gonads, and thus testosterone, from embryos yields the female phenotype (Jost 1972). It is also the precursor to the main female hormone, estrogen. In females, estrogens are the main gonadal hormones that only begin to be secreted during puberty and fluctuate with progesterone throughout the menstrual cycle.

Other sexual differences, specifically brain dimorphisms, are influenced by a third factor, genetics. These differences are direct actions of sex chromosome genes and are not mediated by gonadal hormones (Quinn et al. 2007). Examples of these dimorphisms include aggression, parenting, and habit formation as well as susceptibility to disease (Arnold & Chen 2009).

Relationship Between Estrous Cycle and Alcohol

The female reproductive system consists of the hypothalamus, the pituitary gland, and the ovaries, all of which make up the female hypothalamic-pituitary-gonadal axis (HPA) (Emanuele, Wezeman, & Emanuele 2002). During puberty, increased HPA activity triggers the release of hormones responsible for sexual maturation and growth, including estradiol and progesterone. After menopause, estrogen production continues but is markedly lower and synthesized from androgens, such as testosterone, from the adrenal glands as opposed to the ovaries (Emanuele, Weseman & Emanuele 2002). Similar to humans, female rodents are under the influence of the same ovarian hormones, specifically estrogens and progestagens (Becker et al. 2005), which fluctuate during the estrous cycle and during pregnancy.

In humans, the menstrual cycle can be divided into three phases, 1) Follicular phase, where estrogen and progesterone levels are low, 2) Periovulatory phase, where estrogen peaks and declines and progesterone begins to increase, and 3) Luteal phase, estrogen levels are moderate and progesterone levels are high. Menstruation begins around 10-11 years of age, and each cycle lasts about 28 days (Emanuele, Wezeman & Emanuele 2002).

Rodent cycles are much shorter, about 4-5 days, and are referred to as estrous cycles as opposed to menstrual cycles, due to the lack of shedding of the uterine lining (Becker et al. 2005). The estrous cycle is divided into four phases, 1) Proestrus, where estrogen levels are the highest, and progesterone increases rapidly to a peak then begins to decrease, 2) Estrus, estrogen and progesterone levels decline, 3) Metestrus, estogen levels are low and progesterone levels begin to rise, and 4) Diestrus, estrogen levels rise and progesterone levels decline. It is during the estrus phase that females are sexually receptive and ovulation occurs (Becker et al. 2005).

In rodents, ovarian cyclicity has been shown to produce changes in behaviors such as reproductive behavior, food intake, fluid intake, and locomotor behavior (Eckel, Houpt & Geary 2000). During estrus, females have been shown to have lowest food intake, and maximal locomotor activity (Eckel, Houpt & Geary 2000). It is also during this time that females exhibit sexually receptive behavior (Hardy 1972). Due to the variance of behavior throughout the estrous cycle, males have traditionally been used to examine alcohol-drinking behaviors in rodent models. Despite this, due to the increased consumption compared to male rodents, recent research has sought to examine the effects of hormone cyclicity on ethanol consumption in female rodents.

Research examining the influence of ovarian hormones on drinking patterns in females has yielded complex and inconsistent findings. Studies in primates and humans have shown that females exhibit decreased alcohol consumption during menstrual and premenstrual phases (Mello, Bree & Mendelson 1986). Other studies have found no change in alcohol intake across menstrual cycle phases in humans (Holdstock & de Wit 2000). Research in rodents has shown that ethanol self-administration was lowest in proestrus and estrus stages (Forger & Morin 1982). Other research that artificially synchronized the estrous cycle in rats found that ethanol consumption was lowest during the estrus phase (Roberts et al. 1998). This study used gonadotropin-releasing hormone to synchronize the cycles, though, which may have had its own effect on ethanol consumption. Ovariectomized rats were shown to decrease ethanol consumption, indicating a possible estradiol influence (Carilhol & Mormede, 2001), but this was concurrent with decreased total fluid intake. Overall, a decrease in estradiol and progesterone has been correlated with a decrease in ethanol consumption, but due to the difficulty of measuring ovarian hormones during the rodent estrous cycle without disrupting it, little preclinical research

has examined the influence of female ovarian hormones on drinking. The research that has been done shows conflicting results.

Despite the inconsistency of measuring alcohol intake as a function of female hormones, it is known that alcohol disrupts ovarian cyclicity in humans and rodents. Alcohol has been shown to disrupt female reproductive function at several stages of life (Mello et al. 1993). Studies have shown that alcohol can have detrimental effects on puberty as well as disrupt normal menstrual cycling in young females. Estrogen levels were decreased up to 2 weeks after moderate drinking in females ages 12-18 (Block et al. 1993). Additionally, female alcoholics are known to have a variety of menstrual disorders and even experience complete cessation of menses and infertility (Wilsnack, Klassen & Wilsnack 1984). Delayed ovulation and failure to ovulate (anovulation) have also been seen in heavy and moderate female drinkers (Mendelson & Mello 1988). Alcohol abuse has even been associated with early menopause (Mello et al. 1993). The disruption in cyclicity can be due to many factors including an acute increase in estrogen and testosterone after alcohol consumption (Emanuele & Emanuele 1997; Sarkola et al. 2001). The effects of alcohol on endocrine functions are complex and depend on the amount consumed, the pattern of exposure, the level of intoxication as well as other medical problems, such as liver damage (Emanuele & Emanuele 1997).

Research in rodent models has similarly demonstrated disruptive effects of alcohol consumption on ovarian cyclicity. Alcohol consumption prior to puberty has shown to lead to ovarian failure in female rats (Van Thiel, Gavaler, & Lester 1978), by disrupting the hypothalamic secretions triggering the onset of puberty (Dees, Skelley & Hiney 2001). This effect was prevented with treatment of an opiate receptor inhibitor, naltrexone (Emanuele et al. 2002). The effect of alcohol on the estrous cycle of rodents has been studied by identifying the

amount of time in each stage as well as length of cycles during chronic alcohol intake. These experiments have shown ethanol-fed rats show a decreased frequency in estrus and proestrus stages and an increase in duration of diestrus and metestrus phases (Sanchis, Esquifino & Guerri 1984; Emanuele et al 2001). Additionally, alcohol fed rats had significantly longer cycles compared to water-drinking controls (Krueger, Bo & Rudeen 1983). It has also been found that high levels of alcohol inhibit ovulation, as seen in humans (Sanchis, Esquifino & Guerri 1984).

Alcohol consumption can alter levels of hormones in females. In rats, alcohol consumption led to a temporary elevation of estradiol, but no change in progesterone (Emanuele et al. 2001). Similar findings have also been found in humans (Mello et al. 1993). Testosterone can suppress the hypothalamic-pituitary unit, which would lead to ovarian cycling disruptions. There is still no consensus, though, on how alcohol specifically disrupts these ovarian secretions.

Relationship Between Testosterone and Alcohol

The male reproductive system is made up of the hypothalamus, the pituitary gland, and the testes (Emanuele & Emanuele 2001). The hypothalamus and the pituitary glands serve only regulatory functions, while the testes produce key hormones such as testosterone, which control male sexual characteristics and behaviors (Emanuele & Emanuele 2001). In males, testosterone begins to be secreted in late gestational and neonatal periods (Cooke et al. 1998). Compared to females who are not exposed to this gonadal hormone until puberty, these hormones have the effect of masculinizing males as early as birth and even in utero (Wilson, George & Griffin 1981). Exposure to these gonadal hormones during this critical period of development can cause permanent sex differences through organizational mechanisms. Because of these organizational effects, it is possible that sex differences in alcohol consumption in humans and rodents may be influenced by permanent changes of hormones during early life.

Alcohol consumption has also been shown to affect male gonadal hormones. Male alcoholics have shown reproductive disruptions such as a testicular atrophy, decrease in sperm count, and decrease in sperm mobility (Maneesh et al. 2006). It has been repeatedly shown that both acute and chronic alcohol administration temporarily decreases testosterone synthesis in humans (Mendelson, Mello & Ellingboe 1977; Johnston et al. 1981), and rodents (Adams et al. 1991; Badr & Bartke 1974). It was also found that there was a significant relationship between dose of alcohol and plasma testosterone level (Badr & Bartke 1974). Plasma concentrations of testosterone have also been shown to decrease during ethanol withdrawal in humans (Ylikahri, Huttunen & Harkonen 1980). This decrease in testosterone has been shown to accompany decreases in luteneizing hormone (LH) and follicle stimulating hormone (FSH) (Maneesh et al. 2006), which are produced by the anterior pituitary gland, and not the testes. Thus, it seems that alcohol's effects on testosterone affect not only the testes. Research that has attempted to identify alcohol's effect on testosterone synthesis by examining its biosynthetic pathway has yielded inconclusive results (Emanuel & Emanuele 2001). Because of the negative feedback system that controls the male reproductive system, it is unlikely that the decrease in testosterone following alcohol consumption is due to a decrease in synthesis. This would result in higher LH and FSH, opposite to what was found. It has been suggested that alcohol disrupts the interaction between the nervous and endocrine systems by disrupting the pituitary gland (Maneesh et al. 2006).

Interestingly, high levels of testosterone lead to higher alcohol consumption. One study using alcohol-preferring and alcohol-nonpreferring rat strains found that alcohol-preferring rats had higher levels of testosterone, indicating a positive association between testosterone and alcohol drinking (Apter & Eriksson 2003). Also, castrated rats given testosterone injections preferred ethanol more rapidly and with a greater magnitude than control castrated rats (Lakoza

& Barkov 1980). Similar findings were found in humans. In a twin study, higher testosterone levels were associated with more frequent intoxication and high density drinking (Eriksson et al. 2005). These findings indicate that high testosterone levels may facilitate high alcohol consumption.

While the sex differences in alcohol consumption indicate hormonal influences, studies using ovariectomies and castration in adulthood have not yielded conclusive results (Levinson et al. 2011). These studies aimed to remove the activational effects of gonadal hormones, which occur during adulthood. Another possibility is that sex differences in drinking patterns are caused by early organizational effects of testosterone. One method to study the organizational effects is by masculinizing the brain of female rodents by neonatal testosterone treatment (Seney et al. 2012). This method attempts to create the same organizational changes during this critical period by injecting testosterone within 24 hours of birth. This method can masculinize the brain and behavior of female mice and rats (Guillamon, Segovia & del Abril 1988; Hisasue et al. 2010). If hormone secretions during this critical period shortly before and after birth are mediating the sex differences in alcohol consumption in adulthood, then neonatal testosterone injections should 'masculinize' the female mice and cause them to drink more. Another method of examining the organizational effects of neonatal hormones is by castrating males shortly after birth. Research using this method found that male mice showed decreased sexual function in adulthood (Quadagno et al. 1975). Inhibiting the testes from secreting testosterone during this period and might demasculinize male drinking patterns in adulthood. This study sought to use the neonatal testosterone method to study the organizational effects in females.

Methods for Research on Sex Differences

There are many ways by which to study sex differences in animal models, which depend on the nature of the sex difference. Due to the varying levels of hormones, studying the effects of a trait that differs as a function of estrous cycle can be difficult. One way of doing this is by taking samples of vaginal cells every day and determining the cyclic pattern of each mouse (Becker et al. 2005). Hormonal changes during the ovarian cycle result in cellular changes in vaginal cytology, thus, by examining the vaginal epithelial cells, the hormone concentrations can be estimated (Becker at al. 2005). These vaginal smears can also give information on the effect of drugs or alcohol on the estrous cycle. Using this method, it was shown that alcohol consumption disrupts the normal cyclic pattern of female mice (Sanchis, Esquifino & Guerri 1984; Emanuele et al 2001).

A method of determining if gonadal hormones or another biological system influences a sexually dimorphic trait, is by removal of the gonads by gonadectomy (Berthold 1849). If this is done in adulthood, it is examining the activational role of the hormones. Once gonads are removed, replacement hormone therapy can be administered to determine if the trait returns.

The organizational effects of hormones are due to exposure to hormones during sensitive periods, often in early life (Lenz et al. 2012). One method of examining these effects is by masculinization of females by neonatal testosterone treatment. This method attempts to mimic the early exposure to testosterone in female mice by injecting testosterone within the first 24-hours of birth, and examining its effects in adulthood. The converse experiment is to administer an androgen blocker in utero, and removing the male testes soon after birth. This reduces all exposure to testosterone during the critical period in males.

Past research found that neonatal testosterone injections do not completely masculinize the female rodents, though, as they still contain female genitalia. This is due to some testosterone

being secreted by males in utero. However, different systems have different sensitive periods. The determination of the reproductive tract and of external genitalia is earlier than the brain. Injecting the pregnant mother with testosterone propionate has been shown to masculinize female embryos (Young, Goy & Phoenix 1964). The females then displayed male copulatory behavior as well as decreased responsiveness to female hormones (Phoenix et al. 1959). These injections did not produce any differences in the male embryos.

Animal Models of Alcohol Research

Human disorders are often studied using animal models due to the evolutionary relationship between humans and other animals (Tabakoff et al. 2000). Animal models of research seek to simplify complex disorders, such as alcoholism. Although nonhuman primates are more closely related to genetically and evolutionarily to humans, this research is often costly and difficult to undertake. Rodent models of research offer an alternative and have proven efficient in treatment of disease. These rodent models have been used to study many aspects of alcoholism such as dependence, tolerance, organ damage, and withdrawal.

Alcoholism is a causal factor in more than 60 major types of diseases (WHO 2011). Research has long attempted to shed light on these disorders using both human and animal models. Alcoholism includes symptoms such as 1) cravings, 2) loss of control, characterized by repeated binge phases, 3) physical dependence characterized by withdrawal symptoms that include shakiness or tremors, and 4) tolerance, which is marked by the increased amount of alcohol needed to reach the same effects (NIAAA). Animal models of research have proved useful in understanding the reinforcing and pharmacological effects of alcohol (Lankford et al. 1991, Lopez et al. 2012, Becker et al. 2004), as well as the genetic factors that play a role in the development of alcoholism (Tabakoff & Hoffman 2000). Animal models aim to mimic the

different aspects of human alcohol addiction such as craving, relapse, and binge drinking (Spanagal 2000). While there are differences in alcohol consumptions between humans and rodents, alcohol reinforcement is mediated by subcortical structures, which have been maintained throughout evolution (Spanagal 2000). The similarity in these brain structures does allow for the study of the neurological basis of alcohol consumption and addiction.

There are many methods to study alcohol dependence and in rodents, all of which depend on the aspect of alcoholism. The mode by which to administer alcohol can be through vapor chambers, injection, or voluntary consumption with or without food and water restrictions. To examine the mechanisms underlying chronic alcohol drinking and tolerance, voluntary consumption offers a high level of external validity. Selective breeding has produced strains of mice with high and low consumption patterns (McBride & Li 1998). While rodents will voluntarily drink low concentrations of alcohol, only a few strains are known to prefer it. Strains or mice and rats, including C57BL/6 mice, have been selectively bred and are characterized by high alcohol consumption. (McClearn & Rodgers 1959; Dole, Ho & Gentry 1985; Lankford et al. 1991, Hwa et al. 2012). This preference for ethanol is even seen when other palatable liquids are available, as well as in the absence of sweeteners (Lankford et al. 1991). Addiction incorporates many other behaviors and other models have been used to examine the other aspects such as reinforcement, reinstatement, binge drinking, and withdrawal.

Intermittent Access Alcohol

The intermittent access alcohol procedure is more similar to social drinking in humans than other models by allowing the animals to choose between alcohol and water without having food restrictions (Wise 1973). It is also characterized by alternating phases of binge drinking and abstinence, which are commonly seen in alcoholism and alcohol dependence (Le Magnen 1960).

The intermittent access alcohol procedure is characterized by giving free access to alcohol and water three days a week with just water given on the subsequent days. This results in higher amounts of alcohol consumption as well as higher preference compared to when alcohol is available continuously (Wise 1973; Simms et al. 2008; Hwa et al. 2011; Levinson et al. 2011). This increase in preference and consumption is mediated by repeated cycles of excessive driving followed by abstinence (Sinclair & Senter 1968). These high levels of drinking lead to dependence, which can be characterized by withdrawal assessments, as well as higher blood ethanol levels (Becker & Lopez 2004). This procedure gives insight into the transition from sporadic use to excessive drinking as well the influence of tolerance to the aversive effects of ethanol (Becker & Lopez 2004).

C57BL/6J Mice

Inbred mouse strains are commonly used in alcohol research. These strains exhibit high and low ethanol preference and consumption. The two most commonly used strains are C57BL/6J mice and the DBA/2J mice, which are known for high and low ethanol consumption respectively (Shelton & Grant 2002). The C57BL/6 strain of mice was selectively bred and show high alcohol consumption and decreased sensitivity to the aversive taste of ethanol (Lankford et al. 1991; Hwa et al. 2011). Males are known to consume 20g/kg in 24 hours and females up to 30g/kg in 24 hours, in the intermittent access to ethanol protocol (Hwa et al. 2011). The strain is known to have the same genetic background, facilitating the comparisons between different research laboratories (JAX, 2011).

Aims and Objectives

The first goal of this experiment was to examine the organizational effects of testosterone on drinking patterns in adulthood. Past research examining the activational effect of male and

female gonadal hormones yielded inconclusive results, suggesting a possible early-life effect of male hormones, specifically testosterone, on mediating the sex difference seen in alcohol consumption. The second goal was to further characterize the relationship between female ovarian hormones and alcohol consumption.

Methods

Animals and Housing

Male and female C57BL/6J mice originating from the Jackson Laboratories (Bar Harbour, ME) were bred in house. At post natal day (PD) 60, they were housed individually in polycarbonate cages (28 x 17 x 12 cm) with stainless steel wire mesh lids and pine shaving bedding. Additional male and female C57BL/B6 mice (Jackson Laboratories, Bar Harbour, ME) were 8 weeks upon arrival and were allowed to habituate to the vivarium for one week before beginning the intermittent access procedure. Tap water and standard rodent chow (LabDiet 5001 Rodent Diet; PMI Nutrition International, Brentwood, MO) were available ad libitum. The vivarium maintained a 12-hour reverse light/dark cycle (lights off at 7:00 am, lights on at 7:00 pm) with constant temperature (21±2°C) and humidity (25%). All procedures were approved by the Tufts University Institutional Animal Care and Use Committee and were in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Ethanol Intake Procedures

Fluids were given in 50-ml plastic centrifuge tubes (Nalgene) capped by #5 rubber stoppers (Fisher Scientific, Agawam, MA) with stainless steel ball-bearing sipper tubes (Ancare Corp., Bellmore, NY). Two drinking bottles were placed through the lid concurrently. Tap water was given for 3 days prior to alcohol drinking procedure to allow the animals to acclimate to the bottle arrangement. Ethanol solutions (w/v) were prepared using 95% ethyl alcohol (Pharmaco-

AAPER, Brookfield, CT) and water. Bottles were presented daily beginning 3 hours into the dark cycle and weighed to the nearest hundredth of a gram 24 hours after fluid presentation. An additional 'drip' cage, containing bottles but no animal, was also used to control for accidental spillage, handling, or evaporation. Weekly drip averages were subtracted from each animal's fluid intake. Animals were weighed to the nearest tenth of a gram 3 days per week prior to alcohol drinking in order to calculate grams of alcohol consumed per kilogram of body weight.

Intermittent Access 20% Ethanol Drinking

Mice were given intermittent access to 20% ethanol (Simms et al., 2008; Hwa et al., 2011; Wise 1973). Ethanol was presented in a two-bottle choice (with water) on Monday, Wednesday, and Friday, with free access to water on the remaining days. Prior to the 20% ethanol, mice were given a one-week acquisition period during which 3%, 6%, and 10% (w/v) ethanol was presented on alcohol days. For the remainder of the experiment, mice were given one bottle of 20% ethanol and one bottle of water for 24 hours. Bottles were removed and weighed at the same time the next day, and replaced with water for the following 24 hours. Ethanol bottles were washed and their placement was alternated each drinking session to avoid side preference.

Testosterone Injections

Within 24 hours of birth pups were injected with 0.03mL of either vehicle (sesame oil) or testosterone propionate (100 μg) s.c.. Mice were maintained on standard laboratory diet and water until 8 weeks of age. Female mice injected with testosterone as well as both male and female oil treated mice were then started on the intermittent access to alcohol procedure described above.

Estrous Cycle Monitoring

Stage of estrous cycle of female mice was monitored daily by vaginal lavage using an eyedropper and distilled water. Monitoring began prior to alcohol procedure and continued throughout the acquisition and maintenance period. Samples were collected daily at the same time after bottles were removed for weighing. The eyedropper was filled with a small amount of water and the tip was inserted into the vagina. The water was expelled and then aspirated and placed on a glass microscope slide (Fisher Scientific). Smears were collected in 24-hour intervals.

Once slides were dry, they were stained using 0.5% Cresyl Violet for 1 minute and 30 seconds then rinsed with distilled water and allowed to dry once again. Slides were examined under a microscope and stage of estrous cycle was determined based on cell morphology. Estrus was determined by identifying cornified epithelial cells, diestrus was determined by leukocytes, proestrus by nucleate epithelial cells, and metestrus by a mix of leukocytes and cornified epithelial cells. Both metestrus and diestrus were combined in this experiment.

Withdrawal Assessments

After 4 and 8 weeks of intermittent access to alcohol, mice were tested for alcohol dependence by assessing physical reactions to ethanol withdrawal (Goldstein and Pal, 1971). Severity of convulsions during withdrawal of alcohol correlates to level of dependence in both humans and mice (Goldstein and Pal, 1971). Convulsions were elicited by lifting the mouse by its tail and scoring was on a 0-4 scale based on Goldstein and Pal (1973) [0=no withdrawal signs; 1 = tonic convulsion when the mouse is lifted and given a gentle 180° turn; 2 = tonic-clonic convulsion elicited by the gentle spin, or tonic convulsion when lifted without turning; 3 = tonic-clonic convulsions no requiring any spin; 4=violent tonic-clonic convulsion, often continuing after release of the mouse]. Withdrawal severity was measured after ethanol bottles were

removed at the end of the 24-hour period of drinking and continued every 2 hours for 8 hours.

Study Design

Experiment 1 investigated the effect of neonatal testosterone on ethanol consumption in male and female mice. Males (n=6) and females (n=7) injected neonatally with oil were given intermittent access to ethanol. Another group of females (n=14) injected with testosterone neonatally were also given intermittent access to ethanol.

Experiment 2 examined the influence of intermittent access to alcohol consumption on female gonadal hormones. A group of females (n=12) followed the 8-week intermittent access schedule. Additionally a group of females (n=9) given only food and water were monitored. Estrous cycle was monitored daily.

For each of these experiments, ethanol withdrawal assessments were performed after 4 and 8 weeks of 20% ethanol consumption. Blood ethanol concentration was determined by blood samples taken after 8 weeks of ethanol drinking.

Statistical Testing

Descriptive statistics for all measurements, except HIC scores, were taken over the 8 week maintenance period of 20% ethanol consumption and reported as mean ± standard error mean (SEM). HIC scores are reported as the median ± inter-quartile range. Two-way ANOVAs were used to analyze ethanol intake and preference for intermittent access and group data followed by post-hoc Bonferroni tests. A one-way ANOVA was used to analyze the amount of ethanol consumed per estrous stage. A chi-square test was used to analyze the alcohol-drinking and water-drinking estrous cyclicity.

Results

Experiment 1: Group Differences in Intermittent Access Procedure

A two-way ANOVA found a significant difference between groups on body weight of mice, [$F(2,45)=8.318$, $p<0.050$]. Post-hoc tests revealed a significant difference between oil-treated females (20.771 ± 0.791 g) and oil-treated males (25.022 ± 0.733 g), [$t=3.678$, $p<0.050$]. This confirmed that males were heavier than females. There was also a significant difference between oil-treated females and testosterone-treated females (23.932 ± 0.552 g), [$t=3.082$, $p<0.050$]. There was no difference between oil-treated males and testosterone-treated females [$t=1.172$, $p=0.756$].

Neither sex nor timing of maintenance stage had a significant effect on total volume of fluid consumed [$F(2,45)=0.0816$, $p=0.922$]. All groups had roughly the same total fluid intake (Oil-treated Female: 5.341 ±0.309 mL; Oil-treated Males: 5.435 ±0.286 mL; Testosterone-treated Females: 5.494 ± 0.216 mL). There was also no significant difference in total fluid intake over time [$F(2,45)=0.331$, $p=0.720$].

Ethanol Intake

Consumption of 20% ethanol during the maintenance period of drinking was compared in oil-treated males, oil-treated females, and testosterone-treated females on intermittent access schedules. A two-way ANOVA found a significant difference between groups on ethanol consumption [$F(2,21)=3.861$, $p< 0.05$]. Post-hoc tests revealed that oil-treated females drank significantly more (25.215 ± 1.385 g/kg), than testosterone-treated females (20.476 ± 0.907

g/kg), [t=2.752, $p< 0.050$]. Oil-treated males tended to drink less (21.568 ± 1.282 g/kg) than oil-treated females, but this was not statistically significant [t=1.932, p=0.196].

Two-way ANOVA also indicated a significant difference of ethanol drinking over time for all groups [F(1,21)=6.249, $p< 0.050$]. Post-hoc tests revealed that more ethanol was consumed in the first 2-5 weeks (24.293 ± 1.011 g/kg), than in the second 6-9 weeks (20.547 ± 1.011 g/kg) of the maintenance period [t=2.500, $p<0.050$]. There was no significant difference within any of the groups, though.

Preference

Ethanol preference was calculated for consumption of 20% ethanol. A two-way ANOVA revealed no significant difference between groups on ethanol preference [F(2,45)=1.682, p=0.207]. All groups had similar ethanol preference ratios (Oil-treated Females: 0.488 ± 0.0404; Oil-treated Males: 0.553 ± 0.0374; Testosterone-treated Females: 0.468 ± 0.0282).

Ethanol preference was also compared in the first-half (weeks 2-5) and the second-half (weeks 6-9) of the maintenance period. There was also no significant difference in ethanol preference between the first 2-5 weeks (0.506 ± 0.0127), and the second 6-9 weeks (0.494 ± 0.0131) of the maintenance period [F(2,45)=0.367, p=0.695].

Ethanol Withdrawal Scores

Ethanol withdrawal was assessed using handling induced convulsion (HIC) scores. It was not possible to conduct ANOVAs on these data due to the small group number.

Experiment 2: Influence of Female Gonadal Hormones

Estrous cycle stage was monitored for both intermittent alcohol drinking females and water drinking females. For the ethanol drinking group, a one-way ANOVA did not reveal a

significant correlation between ethanol intake and estrous cycle stage [$F(2,18)=2.354$, $p=0.124$], which could be due to the low level of power (0.245). Averages of ethanol consumption in each stage revealed only a slight decrease in ethanol consumption in during the estrus stage (22.699 ± 1.313 g/kg), compared to proestrus (25.640 ± 2.335 g/kg) and diestrus (25.146± 0.941 g/kg), but this was not significant.

Comparison between time spent in each stage of the estrous cycle for alcohol-drinking females and water-drinking females was done using a chi-square test. Percentage of observed time spent in proestrus, estrus, and diestrus for all mice were calculated and compared. Chi-square analysis revealed no significant difference between groups. Alcohol drinking females did spend more time in diestrus (54.6%) compared to water-drinking females (34.1%), but this was not statistically significant.

Discussion

Main Findings

The present study found that neonatal testosterone injections effectively decreased ethanol consumption compared to oil-treated females. Oil-treated females were found to drink more than oil-treated males, confirming past findings, but the difference was not found to be significant. Mean consumption decreased during the second-half of the maintenance period compared to the first-half, due to a food-change. This consumption was accompanied with a decrease in total fluid intake, but no change in ethanol preference between or within the groups. In the second experiment, intermittent alcohol consumption was found to disrupt the estrous cycle, with females spending more time in the diestrus phase compared to water-drinking females, although this difference was not significant.

Experiment 1: Neonatal Testosterone on Intermittent Access Alcohol

The sexual dimorphism in alcohol consumption in C57BL/6J mice using the intermittent access protocol is well known. Previous studies have shown that B6 females drink more than males during intermittent access to ethanol (Hwa et al. 2011; Levinson et al. 2011). Past research examining the activational effects of hormones did not yield conclusive results, prompting this experiment to examine the role of organizational effects by way of neonatal testosterone-injection.

Intermittent Access to Alcohol

There are several methods to study alcohol dependence in animal models. One model that is used to obtain high levels of consumption is the intermittent access protocol. Rats and mice have been found to drink more under intermittent access compared to continuous access to

alcohol (Levinson et al. 2011; Simms et al. 2008). This is thought to be due to the repeated cycles of binge and abstinence (Pinel & Huang 1975). This procedure has also been used in rats, with both males and females consuming escalating amounts of alcohol after successive binge and abstinence phases (Simms et al. 2008). Past research has found that female C57BL/6J mice drank up to 30 g/kg in 24 hours and males up to 20 g/kg (Hwa et al. 2012). In the present experiment, females were found to drink around 25 g/kg and males around 20 g/kg, in 24 hours, which is consistent with past research. It is thought that these mice are consuming more ethanol compared to continuous access to possibly alleviate the negative symptoms associated with acute withdrawal, a behavior common in human alcoholics (Breese et al. 2005). This also shows the alcohol deprivation effect, where repeated periods of ethanol access followed by periods of deprivation leads to an increase in ethanol preference and consumption (Breese et al. 2005; Melendez, Middaugh & Kalivas 2006).

Sex Differences in Alcohol Consumption

Sex differences in alcohol consumption occur in humans and rodent models. The results of this study confirm past research that found that female C57BL/6J mice drink more than males in intermittent access procedures (Hwa et al. 2012), as well as continuous access (Yoneyama et al. 2008). Other animals such as rats also exhibit similar sexual dimorphisms. Female Long-Evans rats also consumed more alcohol per body weight than males (Lancaster et al. 1992). This study found that females drank about 25 g/kg, compared to males, which drank closer to 20 g/kg. This strain of mice is often used in alcohol research due to the high consumption and tolerance to the aversive taste of alcohol. Other studies using this strain of mice found that females drank up to 30 g/kg of ethanol in 24 hours (Hwa et al. 2011). This experiment found slightly lower numbers, which may be due to the change in food.

Female rodents have also been found to have higher preference for ethanol than males (Lancaster et al. 1992). This is inconsistent with this study's findings that B6 males have higher preference ratios than either female group. The difference was not significant, though, and could be due to the low number of animals in the oil-treated male group.

Organizational vs. Activational Effects

Past research has shown that hormones can differentially effect the development of the nervous system. Hormone secretions during adulthood influence behaviors in a transient manner, and are referred to as the activational effects. During early development there are sensitive periods where hormone secretions can influence nervous system development in a permanent manner, which refer to the organizational effects (Lenz et al. 2012; Phoenix et al. 1959).

Because gonadal hormones are thought to play a role in alcohol consumption patterns in adulthood, past research has examined the effect of adult castration and ovariectomies on drinking patterns in C57BL/6J mice. This research showed that castration resulted in a slight decrease in ethanol consumption, and ovariectomy resulted in no change in ethanol consumption (Levinson et al. 2011). Hormone replacement therapy also did not yield any changes in drinking patterns (Levinson et al. 2011). Both of these findings suggest that the activational effects of hormones in adulthood are not mediating the sex differences seen in drinking patterns.

Research has identified a maximally sensitive period for organizational effects of sex hormones in late pre-natal and early post-natal development (Baum 1979; Schulz, Molenda-Figueira & Sisk 2009). Exposure to perinatal testosterone was found to "defeminize" rodents, which resulted in reduced capacity to display female coital behavior after gonadal hormone injections in adulthood (Baum 1979). Another study found that play fighting in rats can be induced in females by neonatal testosterone injections, and removed in males by neonatal

castration (Pellis 2002). Thus, certain traits are mediated by testosterone secretions early on in life. This study found that testosterone secretions within 24-hours of birth critically altered drinking patterns in adulthood. Testosterone-treated females were found to drink less than the control oil-treated females, suggesting that these organizational effects are permanent and long lasting. These injections effectively reduced the amount of alcohol consumed by female mice to commonly seen male levels.

Future studies examining the organizational role of hormones on alcohol consumption could benefit from using other methods. One such method would be to determine the length of this critical period in mice by injecting testosterone on various days after birth. Additionally, it would be interesting to use other doses of testosterone. This study used one dose of testosterone that has been used in other studies (Sternberg et al. 1995). It is possible that a larger dose, or repeated injections could more effectively "masculinize" the female B6 mice and yield more significant data. Additionally, the converse of this experiment would be to attempt to inhibit masculinization in male mice. Neonatal castration may be beneficial to further understand this critical period. If this period in development were responsible for the sexual dimorphism in adulthood, neonatal castration would presumably cause males to drink greater amounts in adulthood. Past research examining the organizational effects of aggressive and sexual behavior compared neonatally castrated males that were given hormone replacement during development and neonatally castrated males who were given hormone replacement in adulthood (Shultz et al. 2006). This research found that males that missed that critical period of testosterone showed low levels of aggressive and sexual behavior.

While it is not as fully understood, female gonadal hormones may have organizational effects on alcohol drinking. Neonatal and pubertal ovariectomies have found to masculinize

females in their food guarding behaviors (Field et al. 2004). While the ovaries are not known to secrete estrogens and progestagens to a large extent until puberty, these findings indicate a complex and interactive nature of steroid-dependent periods on these behaviors across development (Schulz, Molenda-Figueira & Sisk 2009). It might be interesting to examine these perinatal and pubertal effects of ovarian hormones on alcohol drinking in adulthood.

Another method of halting the masculinizing effects of testosterone neonatally is by injection of a testosterone antagonist. This would inhibit the sections of testosterone and presumably yield results similar to neonatal castration. Testosterone is also secreted by the adrenal glands, which may have peripheral influences.

Withdrawal Assessments

Handing-induced convulsions are a measurement of withdrawal from alcohol. Human alcoholics are known to show tremors during abstinence from alcohol (Veatch, Wright & Randall 2007). Thus, as dependence worsens as do the tremors during abstinence. The increased alcohol consumption seen in the intermittent-access protocol could be due to alleviating these symptoms.

In terms of gender differences, it would be expected that females would portray higher levels of withdrawal due to their higher levels of drinking. But results showed that there was no significant difference after 8 weeks of intermittent access between groups. Past research examining the sex differences in HIC scores after 8-weeks of intermittent access to 20% ethanol consumption found that males showed increased handling-induced convulsions compared to females (Levinson et al. 2011; Veatch, Wright & Randall 2007). It is possible that the sex difference in withdrawal is not prominent due to a floor effect.

Withdrawal induced seizures is only one method of examining alcohol dependence and it might be beneficial, at least in this strain of mice, to use other methods of determining alcohol dependence between genders. In humans, delirium tremens, blackouts, and other alcohol related behaviors associated with seizure propensity are more likely in male alcoholics than females (Rimmer et al. 1971). Also, withdrawal susceptibility may be due to differences in brain alterations after exposure to alcohol. Research has found that sex differences in g-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) receptor subunit mRNA and protein levels (Devaud et al. 1996). This suggests that these sex differences in withdrawal may be due to differences in neurotransmission and not necessarily hormonal differences (Allele & Devaud 2006). If this were the case, then neonatal testosterone injections would not have a masculinization effect on withdrawal behaviors.

Other withdrawal-induced behaviors can be used such as enhanced anxiety-like behaviors and heightened startle responses (Overstreet, Knapp & Breeze 2004; Macey et al. 1996). While research inducing alcohol dependence via ethanol diets in rats found no sex difference in anxiety-response using the elevated plus maze (Overstreet, Knapp & Breese 2004), anxiety-like behaviors in mice may be different.

Experiment 2: Influence of Female Gonadal Hormones

There was no effect of estrous cycle stage on ethanol consumption. This is consistent with other research that has found no influence of ovarian hormone levels on drinking patterns (Cailhol & Mormede, 2001; Roberts et al. 1998). There was a trend that chronic alcohol consumption increased the amount of time spent in diestrus phases of the estrous cycle, which is consistent with other research. This was not statistically significant, though. Another method that

could be used is to compare estrous cycling patterns in female mice before and after starting an intermittent alcohol consumption procedure.

Limitations

Statistical testing would have been more convincing if more animals were used in the study. Due to the protectiveness of mice and the relatively low amount of care of C57BL/6J dams, many pups and even entire litters did not survive after oil and testosterone injections. Due to the time constraint and the number of breeding pairs, it was difficult to obtain experimental animals, resulting in low numbers for the oil treated groups. Additionally, the food change that occurred in the middle of the maintenance period decreased the alcohol consumption data, which most likely obscured the statistical results. Although this food change did not effectively change the preference for alcohol, the decrease in total fluid intake seen skewed the values and made it difficult to determine the true values of the statistical differences.

In terms of the testosterone injections, no sealing solution was used, which could have resulted in some solution leaking out. It was of the opinion of the experimenter that a sealing adhesive solution could have made the dam less likely to care for the pups and decreased the survival rate further. Although it is possible that some of the experimental solution did escape the animals, the experimental data suggest that the dose given was sufficient to allow for organizational changes to occur.

Withdrawal can be measured a variety of ways, with handling-induced convulsion (HIC) scores being one of them. While human alcoholics are known to experience tremors during abstinence periods, this behavior can also suggest dependence in mice. Despite this, it seems this strain of mice does not experience high levels of HIC (Levinson et al. 2011), which could contribute to a floor-effect. It would be perhaps beneficial in future studies using C57BL/6J mice

to determine another method of alcohol withdrawal measurements. The low values for all groups make it difficult to discern a difference in behaviors. Additionally, more animals should be added to all groups. This would allow for statistical analysis to determine if the differences between groups and between 4-week and 8-week data were significant.

Future Directions

This study would benefit from the use of blood ethanol concentration measurements. These measurements were taken the day prior to the 8-week withdrawal assessments and could reveal insights into differences in withdrawal assessments. Additionally it could reveal parallels to levels in humans. Additionally, more oil-treated male and oil-treated female subjects should be added to verify statistical testing. This would enable ANOVAs to be performed on withdrawal assessments as well as create more dependable data.

The food change during the maintenance stage created unstable values, which may need to be replicated due to the statistically lower values seen in the second half of the maintenance period. Unfortunately, although the preference remained the same and the decrease in ethanol consumption was concurrent with a decrease in total fluid intake, these values lower the average values.

Other methods of examining the organizational effects of testosterone should be explored. While this experiment made use of one dose of testosterone propionate, varying the doses and examining differential effects on adult drinking could lead to insights into this critical period and the amount of testosterone needed to ‘masculinize’ female mice could be determined. Additionally, several doses of testosterone could be given in succession to deter against leakage. This could make it more difficult for pups to survive, though, which might make varying one single dose preferable. Neonatal castration could be used to determine if the opposite

phenomenon could be seen in male mice. This could also be done in combination with neonatal injections of androgen-blockers.

Assessments on the influence of alcohol on ovarian cyclicity may benefit from characterizing the metestrus stage independent of the diestrus stage. This study grouped them together due to the similarity in hormone levels, but perhaps this is also obscuring differences from control animals. Due to the complexity of this relationship, it may be beneficial to first characterize alcohol's effects on ovarian hormones to determine an improved method of examining female gonadal hormone effects on alcohol consumption.

Concluding Results

In summary, this present study suggests an influence of organizational effects on alcohol consumption in adulthood. Neonatal secretions of testosterone seem to lead to be responsible to the sex differences in ethanol consumption. Future investigations into this phenomenon would benefit from neonatal castration, which would presumably lead to an increase in alcohol consumption in male mice. These effects would give more insight into the permanent effects of these hormones, and might be useful in understanding the epidemiology of alcohol dependence in humans. This is especially important considering the decrease in the gender gap in alcohol-dependence and abuse in humans in recent years.

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Table Captions

Table 1. Study design for the present experiment. Acquisition phase consisted of one week of 3%, 6%, and 10% ethanol followed by 8 weeks of 20% ethanol in the maintenance phase. HIC tested was done on water-drinking days after 4 weeks and 8 weeks of 20% drinking.

Table 2. Body weight and volume (mL) consumed of 20% ethanol, water, and total fluid are represented as mean ± SEM for the 8 week maintenance period in intermittent access oil-treated female, oil-treated male, and testosterone-treated female mice. * $p<0.05$ difference between groups

Table 3. Body weight and volume (mL) consumed of 20% ethanol, water, and total fluid are represented by mean ± SEM for the 8 week maintenance period in the intermittent access female estrous cycle mice.

Table 4. Median and inter-quartile range for handling-induced convulsion (HIC) scores after 4-weeks of intermittent access to 20% ethanol. Control females include estrous monitored females from Experiment 2 and oil-treated females from Experiment 1. * $p<0.05$

Table 5. Median and inter-quartile range for handling-induced convulsion (HIC) scores after 8-weeks of intermittent access to 20% ethanol. Control females include estrous monitored females from Experiment 2 and oil-treated females from Experiment 1.

Figure Captions

Figure 1. Weekly averages of ethanol intake (g/kg) for males and female C57BL/6J mice given oil injections (pink: females, blue: males) or testosterone injections (green: females) during intermittent access to ethanol. Data are mean weekly group intake ± SEM. For testosterone-treated females n=14, for oil-treated females n=6, and for oil-treated males n=7. Concentration of ethanol increased from 3%, 6%, and 10% during the first week of acquisition, and remained at 20% during the 8-week maintenance period.

Figure 2. Average of ethanol intake (g/kg) for male and female C57BL/6J mice given oil injections (pink: females, blue: males) or testosterone injections (green: females) during the first 4 weeks and second 4 weeks of the maintenance period. Data are mean group intake ± SEM. * p<0.05 difference between timing of maintenance period.

Figure 3. Total average of ethanol intake (g/kg) for male and female C57BL/6J mice for the entire 8-week maintenance period. Data are mean group intake ± SEM. * p<0.05 difference between testosterone-treated females and oil-treated females.

Figure 4. Preference ratios of male and female mice receiving intermittent access to 20% ethanol over the first 4 weeks and second 4 weeks of maintenance period. Pink represents oil-treated females, green represents testosterone-treated females, and blue represents oil-treated males. Data are mean group preference ratio ± SEM.

Figure 5. Handing-induced convulsion (HIC) scores after 4-weeks of intermittent access to 20% ethanol for control females, control males, and testosterone females. Control females include oil-treated females from Experiment 1 and estrous monitored females from Experiment 2. Data a median ± interquartile range.

Figure 6. Handing-induced convulsion (HIC) scores after 8-weeks of intermittent access to 20% ethanol for control females, control males, and testosterone females. Control females include oil-treated females from Experiment 1 and estrous monitored females from Experiment 2. Data a median ± interquartile range.

Table 1. Study Design

		Ethanol Drinking		
		Week 1	Week 2-9	
Intermittent Access	Oil-Males (n=7)	Acquisition	Maintenance	HIC + BEC
	Oil- Females (n=6)	Acquisition	Maintenance	HIC + BEC
	Testosterone-Females (n=14)	Acquisition	Maintenance	HIC + BEC
Intermittent Access				
	Estrous Females (n=12)	Acquisition	Maintenance	HIC + BEC

Table. 2. Body weight and fluid consumption of C57BL/6J mice

	Body Weight (g)	**Ethanol (mL)**	**Water (mL)**	**Total (mL)**
Oil-treated Females	20.77±0.79 *	2.60±0.22	2.84±0.19	5.341±0.31
Oil-treated Males	25.02±0.73 *	2.96±0.29	2.51±0.27	5.435±0.29
Testosterone-treated Females	23.93±0.55 *	2.39±0.12	3.12±0.24	5.494±0.22

Table 3. Body weight and fluid consumption of C57BL/6J mice

	Body Weight (g)	**Ethanol (mL)**	**Water (mL)**	**Total (mL)**
Estrous Monitored Alcohol-Drinking Females	21.07±0.36	2.46±0.12	1.56±0.15	4.024±0.16

Table 4. Median and interquartile range for handling-induced convulsion (HIC) scores after 4-weeks of intermittent access to 20% ethanol.

Group	**N**	**Median**	**25%, 75%**
Control Females	18 12- Estrous Experiment Females 6- Oil-treated Females	0-hour: 0	0-hour: 0,0
		2-hour: 0	2-hour: 0,0
		4-hour: 0	4-hour: 0,0
		6-hour: 0	6-hour: 0,1
		8-hour: 1	8-hour: 0,1
Control Males	7	0-hour: 0	0-hour: 0,0
		2-hour: 0	2-hour: 0,1
		4-hour: 0	4-hour: 0,1
		6-hour: 1	6-hour: 1,1
		8-hour: 1	8-hour: 0,2
Testosterone Females	3	0-hour: 0	0-hour:0,0
		2-hour: 0	2-hour: 0,0
		4-hour: 1	4-hour: 0,1
		6-hour: 1	6-hour: 1,2
		8-hour: 2	8-hour: 0,2

Table 5. Median and inter-quartile range for handling-induced convulsion (HIC) scores after 8-weeks of intermittent access to 20% ethanol.

Group	**N**	**Median**	**25%, 75%**
Control Females	15 11- Estrous Experiment Females 4- Oil-treated Females	0-hour: 0	0-hour: 0,0
		2-hour: 0	2-hour: 0,1
		4-hour: 0	4-hour: 0,1
		6-hour: 1	6-hour: 0,1
		8-hour: 1	8-hour: 1,2
Control Males	3	0-hour: 0	0-hour: 0,1
		2-hour: 1	2-hour: 0,1
		4-hour: 0	4-hour: 0,1
		6-hour: 1	6-hour: 0,2
		8-hour: 1	8-hour: 0,2
Testosterone Females	10	0-hour: 0	0-hour: 0,0.25
		2-hour: 0	2-hour: 0,1
		4-hour: 1	4-hour: 0,1
		6-hour: 1	6-hour: 0,1.250
		8-hour: 1	8-hour: 1,2

Figure 1.

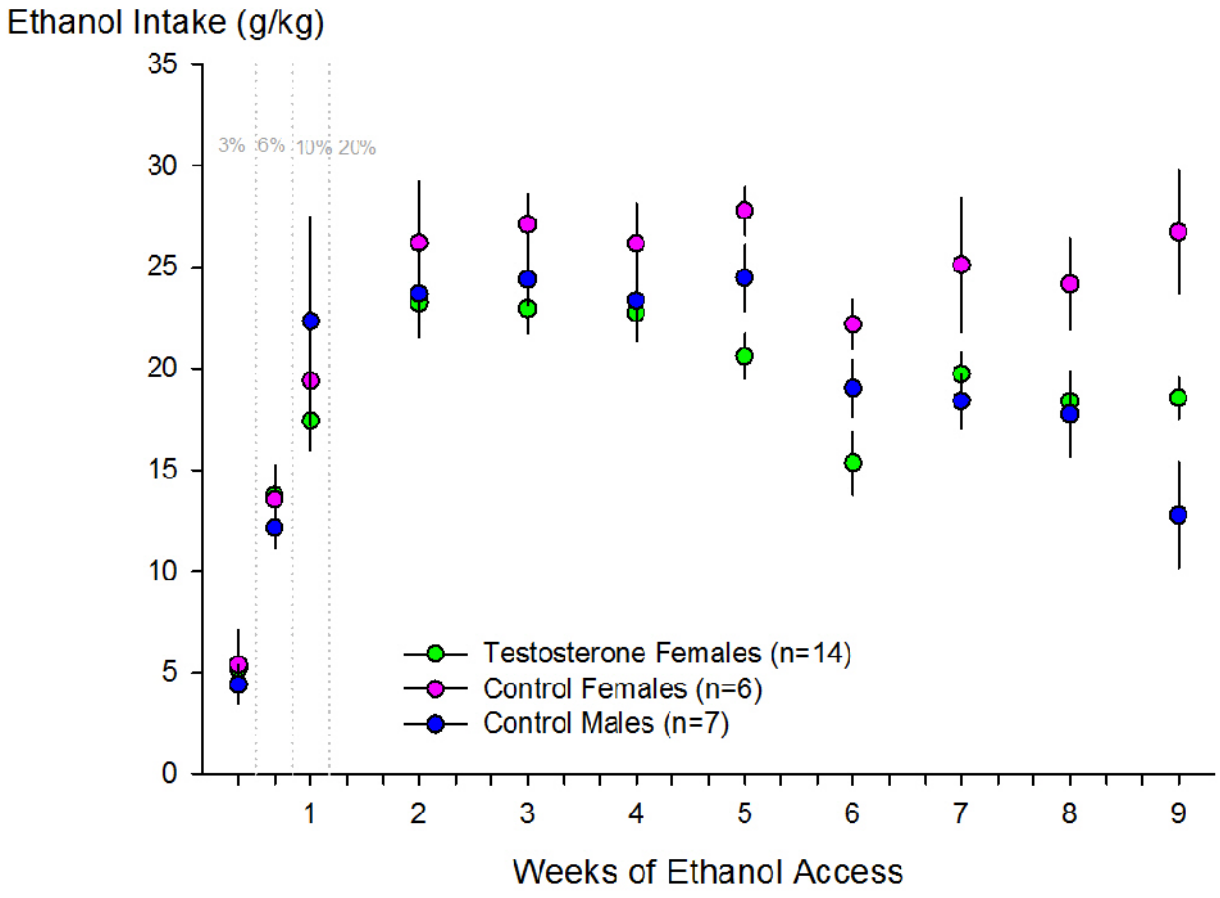

Ethanol Intake (g/kg)
35
30
25
20
15
10
5
0
3%
6%
10%
20%
Testosterone Females (n=14)
Control Females (n=6)
Control Males (n=7)
1
2
3
4
5
6
7
8
9
Weeks of Ethanol Access

Figure 2.

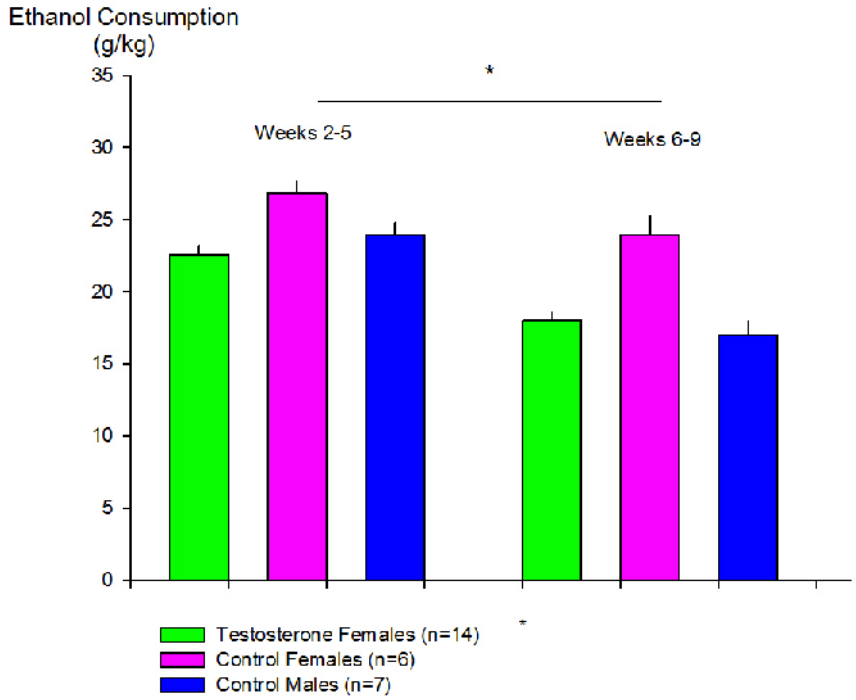

Ethanol Consumption
(g/kg)
35
30
25
20
15
10
5
0
*
Weeks 2-5
Weeks 6-9
*
Testosterone Females (n=14)
Control Females (n=6)
Control Males (n=7)

Figure 3.

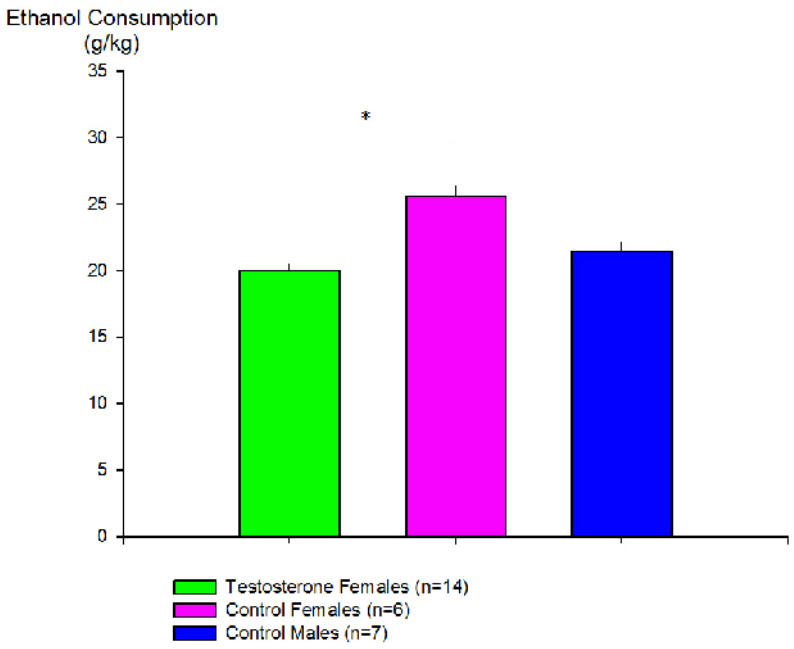

Ethanol Consumption
(g/kg)
35
30
25
20
15
10
5
0
*
Testosterone Females (n=14)
Control Females (n=6)
Control Males (n=7)

Figure 4.

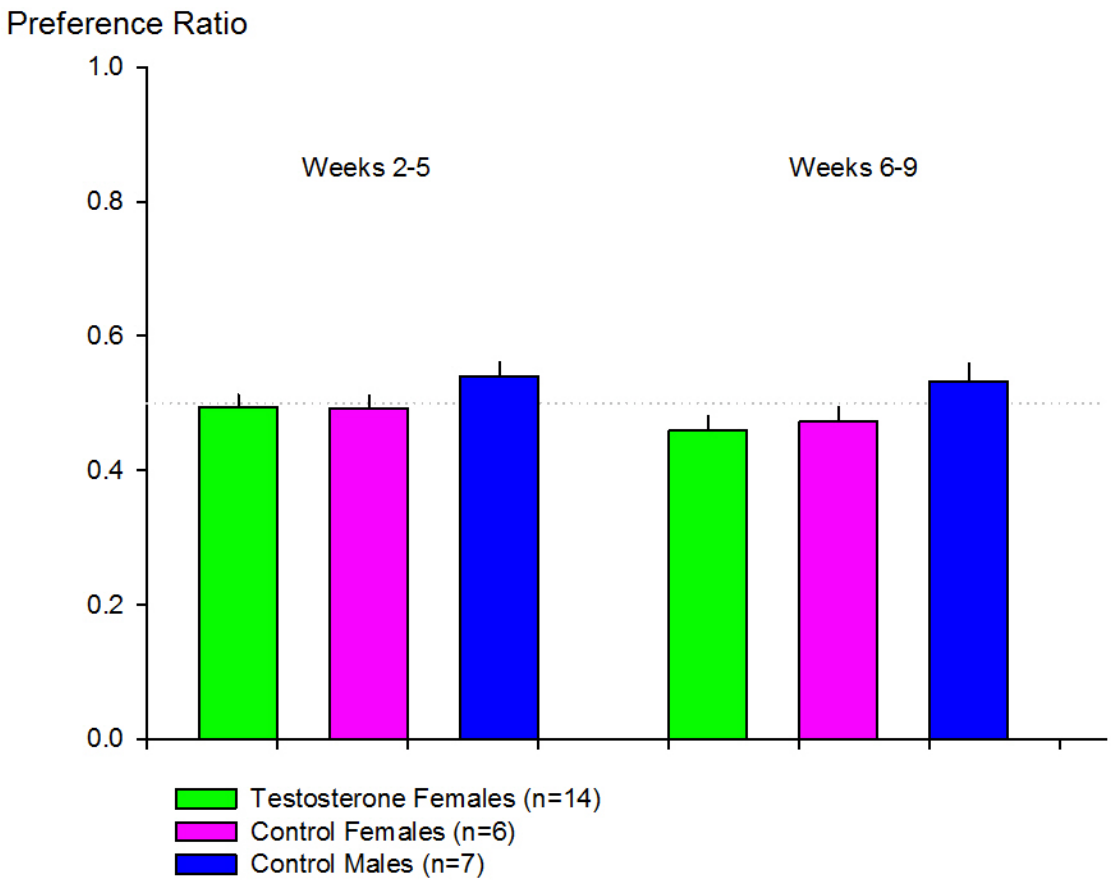

Preference Ratio
1.0
0.8
0.6
0.4
0.2
0.0
Weeks 2-5
Weeks 6-9
Testosterone Females (n=14)
Control Females (n=6)
Control Males (n=7)

Figure 5.

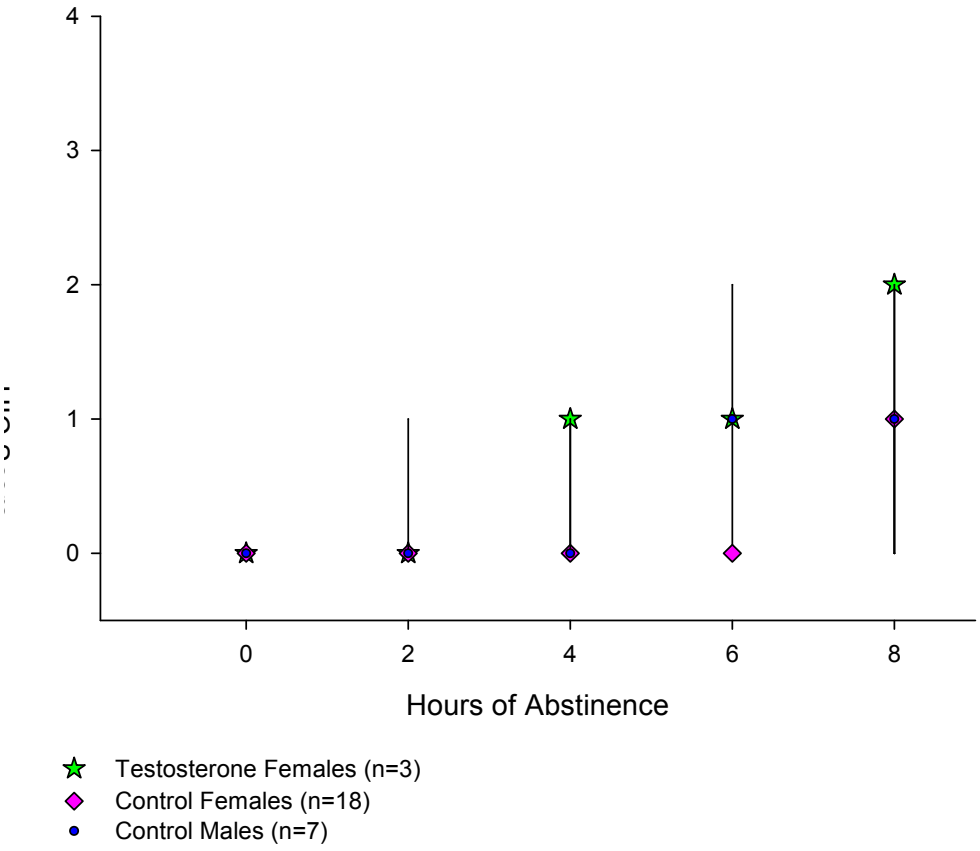

4
3
2
1
0
0
2
4
6
8
Hours of Abstinence
Testosterone Females (n=3)
Control Females (n=18)
Control Males (n=7)

Figure 6.

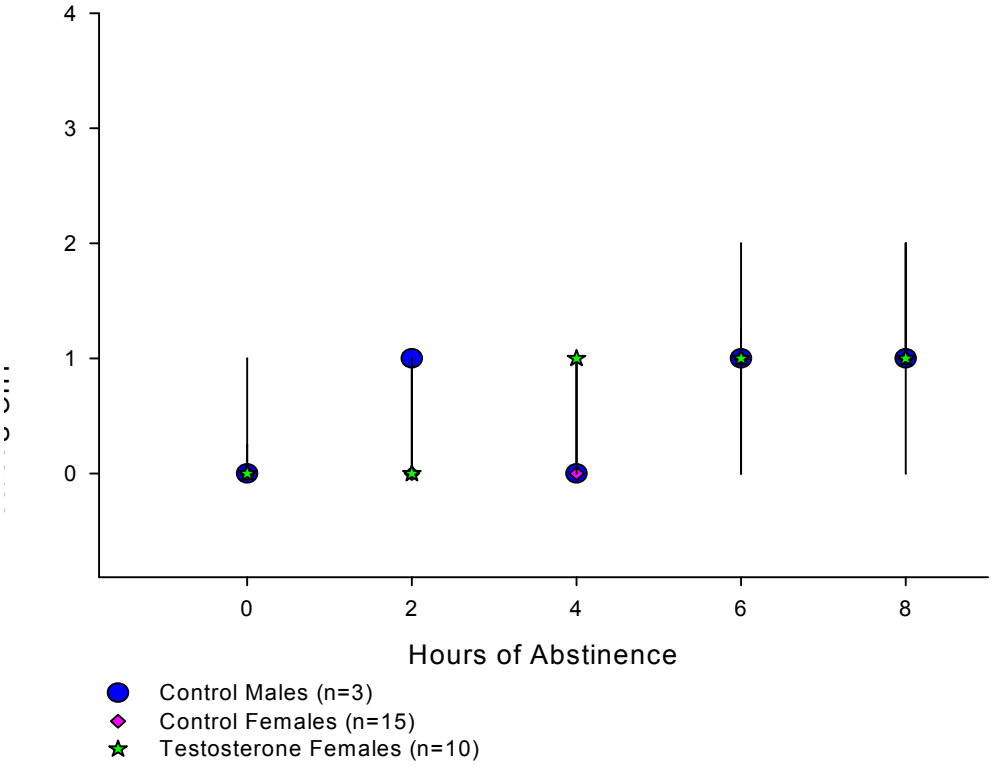

4
3
2
1
0
0
2
4
6
8
Hours of Abstinence
Control Males (n=3)
Control Females (n=15)
Testosterone Females (n=10)

Figure 7.

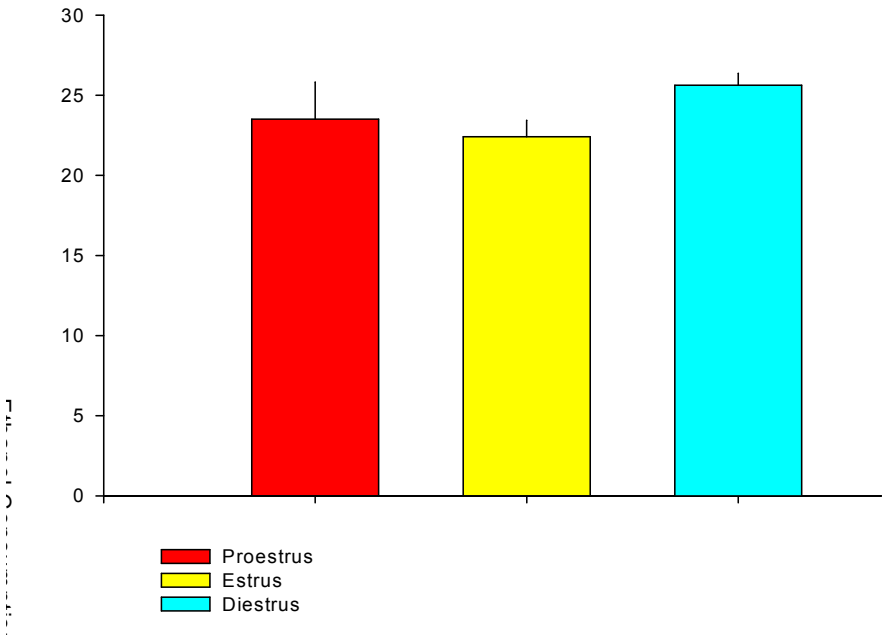
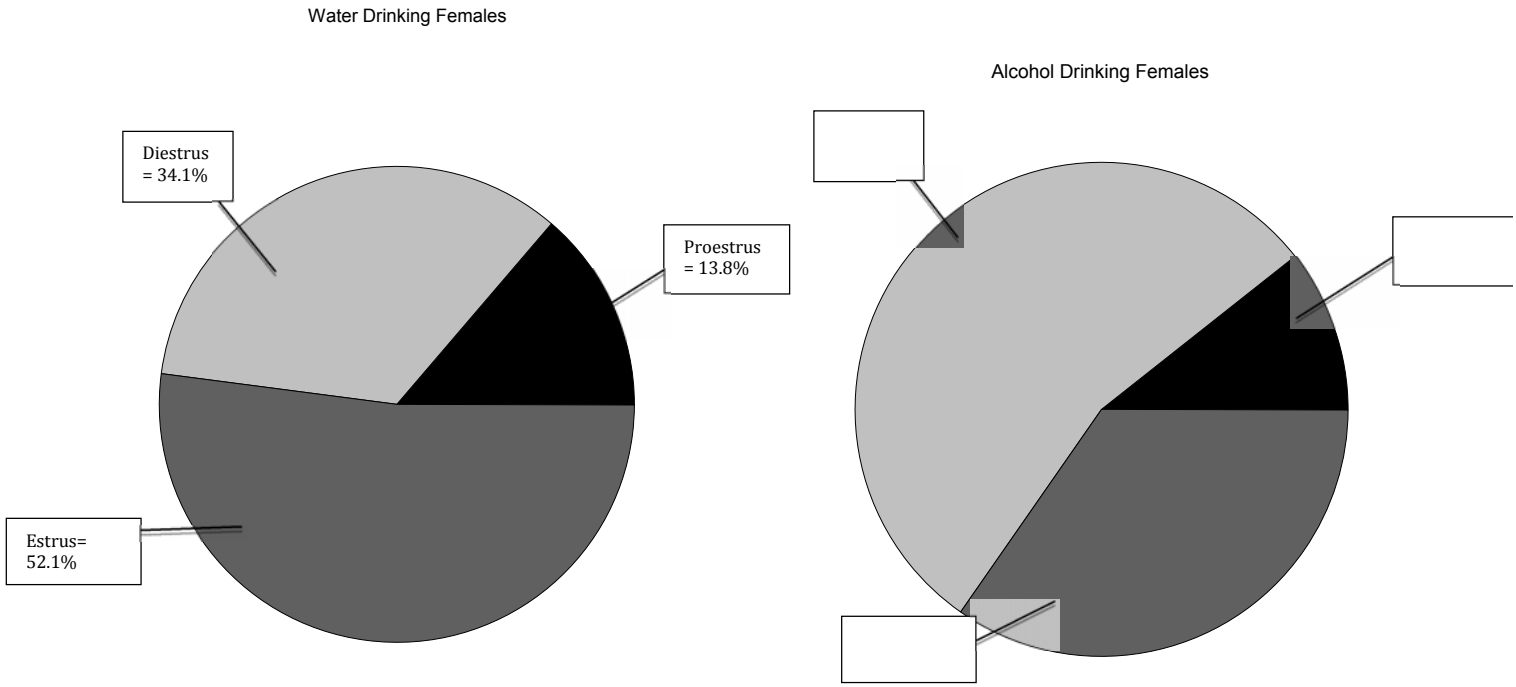


Figure 8.


Water Drinking Females
Diestrus
= 34.1%
Proestrus
= 13.8%
Estrus=
52.1%
Alcohol Drinking Females