A swine model for Fetal Alcohol Spectrum Disorder – The effect of maternal free-choice ethanol consumption on the behavior of female offspring

by

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A Dissertation

In

Animal Science

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

Doctor of Philosophy

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ACKNOWLEDGMENTS

First I would like to thank Dr. John McGlone for asking me three years ago to get my Ph.D. under him. Without his belief that I was a good scientist and researcher I may not have had this great opportunity. Thank you for including me in every aspect of research and the business of research. I believe my graduate experiences have been unique and well rounded, and I feel you have prepared me to have a successful career. I have learned so much from you, and know that I have plenty more to learn. I know you will be a lifelong supporter to help me to succeed professionally any way you can. I also want to thank you for allowing me to do this research because I know it was unfunded and financially draining. I appreciate having this opportunity to expand on the many uses of pigs as biomedical models.

I would also like to thank my committee members, Drs. Mhairi Sutherland, Tiffanie Brooks, Susan Bergeson and Peter Syapin for their guidance. Dr. Sutherland has been an amazing mentor and friend even many miles away for several years now. I owe my passion for research to her, and I would not have ever gone to graduate school if I had not started working for her, and Dr. Brooks' friendship and encouragement over the years has helped keep me sane. Dr. Bergeson and Syapin I could not have done this project without your knowledge, advice, guidance and mentoring. This is a completely new subject for me, and I know I haven't even begun to understand the depths of alcohol research. I thank you for your patience and time in helping me to learn this fascinating area of work.

Thank you to my lab mates and fellow graduate student friends past and present. It is nice having friends to suffer through the grad school grind with! I could not have done all this research without the help of all the student workers the past few years, and there are a few of you. I know it is boring watching videos all day, but I am so appreciative of the help in the lab and at the farm. I would still be back there in the behavior lab watching videos today and would never graduate without your help. I also thank Stanley and Edward at the farm for helping me on a daily basis with my studies and accommodating every weird request I had.

I cannot thank my wonderful husband enough for his support and encouragement. He is my best friend and biggest supporter, and his encouragement when I lose faith in myself has kept me going and helped me to believe in my capabilities. I have such an amazing family and support system between my parents, in-laws and all the family and friends in between. There is so much love in my life and I will never be able to thank you all enough for your belief and support in my long career goals. I have the greatest fans in the world.

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ABSTRACT

Pigs are a great biomedical model and are very similar to humans in anatomy, physiology, and behavior. Antidotal stories have always portrayed pigs as a species that will freely drink and get intoxicated, and the pig has similar ethanol pharmacokinetics as humans. Therefore, we used the domestic pig as a novel model for Fetal Alcohol Spectrum Disorder.

Pig ethanol pharmacokinetics are similar to the human, but this has only been shown in younger pigs. In order to show the ethanol absorption, distribution and elimination of ethanol in a model that represents oral consumption of ethanol, we intragastrically gavaged pregnant sows with 3 g/kg of 30% ethanol. We found this dose produced high blood ethanol concentrations (BEC) and that pigs were intoxicated. But variability between pigs was high and a long plateau phase was observed in ethanol metabolism. More pigs, better blood collection location, and longer collection period are needed to determine ethanol pharmacokinetics in older, pregnant pigs.

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term that represents the broad range of developmental, behavioral, mental and anatomical deficits caused by the teratogenic effects of ethanol consumption during pregnancy. Many factors influence the severity of FASD and how much ethanol reaches the fetus, such as peak blood ethanol concentrations, dose of ethanol, rate of consumption, and timing and pattern of exposure. Our objective was to use a free-choice drinking paradigm in a pig model to evaluate the behavioral implications of offspring prenatally exposed to ethanol. Coping style was assessed by a backtest at 5 d of age. Anxiety was measured in an isolation test (14 d of age), while locomotion, anxiety, and novelty seeking was measured in an open field test (28 d of age). Social status was measured within the litter by teat order (7 d of age), against an unfamiliar pig in the socialization test (16 d of age), and within the home pen by a food competition test (150 d of age). Learning and cognition were assessed in a series of maze tests at 35 d of age.

The effects of prenatal alcohol exposure (PAE) were assessed using a Completely Randomized Design in experiment 1. All pigs remained with the birth sow throughout the lactation period and only two treatments were assessed, control (CON) and ethanol exposed (ETOH). In experiment 2, we used a Randomized Complete Block Design, in which pigs were cross fostered to the opposite rearing treatment or remained with the birth sow; control-control (CC), control-ethanol (CE), ethanol-control (EC) and ethanol-ethanol (EE).

Freely drinking sows consumed an average of 2 g/kg of 20% ethanol the first 10 wk of gestation which did not affect the litter sizes and numbers of pigs born dead or alive, or offspring weights at birth, weaning and harvest, but differences in behavior tests were observed. Prenatal exposure to ethanol made ETOH pigs in experiment 1 low responders to stress as assessed by the backtest, but EE pigs in experiment 2 were higher responders to stress. Isolation was more fearful for CON pigs and a novel environment and object elicited a larger fear response for CON and EC pigs. Ethanol pigs were more active in the open field test. Ethanol pigs were quicker at completing the mazes and more pigs successfully completed them compared to CON pigs regardless if more errors were made. Prenatal exposure increased aggression and therefore social status at an early age, but at adolescence this was reversed and ETOH pigs were more submissive. The submissive social status and PAE at adolescence increased ethanol consumption in EE pigs when individually housed. Behavioral findings in the pig model are similar to FASD characteristics in humans.

Differences between treatments in both experiment 1 and 2 indicate prenatal ethanol treatments as well as lactation rearing environment influenced the behavior of pigs, and that the pig could be a good model for FASD. However, this is the first research of its kind, and more needs to be completed to develop a replicable pig model.

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ACTH	adrenocorticotropin hormone
ADHD	Attention – Deficit/Hyperactivity Disorder
BEC	blood ethanol concentration
CBCL	Child Behavior Checklist
CNS	Central Nervous System
CON	control
ETOH	ethanol treatment of sows and piglets
FAE	Fetal Alcohol Effect
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorder
fMRI	functional Magnetic Resonance Imaging
HPA	Hypothalamic Pituitary Adrenal axis
HR	high responder in the backtest
IR	intermediate responder in the backtest
LR	low responder in the backtest
MRI	Magnetic Resonance Imaging
N:L	neutrophil to lymphocyte ratio
OB	oxidative burst
PAE	prenatal alcohol exposure
VABS	Vineland Adaptive Behavior Scales

CHAPTER I

LITERATURE REVIEW

What is FASD

Fetal Alcohol Spectrum Disorder (FASD), the umbrella term that represents the broad range of developmental, behavioral, mental and anatomical deficits that present in the offspring of mothers who heavily drank during pregnancy (Riley and McGee, 2005), is the leading cause of mental retardation and birth defects in children (Ismail et al., 2010; Thackray and Tifft, 2001). A teratogen is any factor (genetic, environmental or a combination) that causes abnormal fetal development and leads to birth defects. The abnormalities may be on the surface of the body or internal, and although the anatomical abnormalities are present at birth they may not be diagnosed until later in life (Chung, 2005). Alcohol is the most common human teratogen, but drinking alcohol during pregnancy is also a preventable teratogen.

However, not every child prenatally exposed to alcohol results in the same degree of developmental challenges. Some children are not affected at all or to a very low degree, and are diagnosed with Fetal Alcohol Effect (FAE), or they may have distinct facial anomalies that are present in diagnosis with Fetal Alcohol Syndrome (FAS).

At the severe end of the spectrum, FAS includes three distinct areas of anomalies: prenatal or postnatal growth deficiency, facial anomalies, and central nervous system (CNS) dysfunction (Jones and Smith, 1973). The distinct facial features include: microcephaly, flat midface, and a poorly developed philtrum and thin

upper lip. CNS impairment includes neurological abnormalities, developmental delay, hyperactivity, or intellectual and cognitive impairment (Jones and Smith, 1973; Thackray and Tifft, 2001). There can be evidence of prenatal alcohol exposure, but if all three anomalies are not present, then the child lacks the criteria for FAS diagnosis.

Although efforts have been made to refine and better quantify the degree of anomalies required to diagnose FAS, no efforts have been made to address the issue of quantifying maternal ethanol use (Riley and McGee, 2005) because this information comes from self-reports of alcohol use. Women may drink equivalent amounts of ethanol during pregnancy and some will have babies without FASD anomalies, while others will have children with more severe symptoms.

One of the most influential factors that lead to the spectrum of variation in prenatal ethanol exposure is the amount of alcohol that actually reaches the fetus (Riley and McGee, 2005), and peak blood ethanol concentrations (Thackray and Tifft, 2001) determined by the dose, rate of consumption, and pattern of the alcohol exposure. Peak blood ethanol concentration (BEC) is a risk factor in determining FASD severity. When ethanol is consumed in a binge-like manner, small daily doses of ethanol can induce high peak BECs and severe damage to the offspring. There are correlations between high BECs and the extent of damage in structural alterations (Bonthius and West, 1990; 1991a, b) and in functional impairment (Goodlett et al., 1987; 1991; 1992).

Timing of exposure relative to fetal developmental milestones determines which structures are affected and to what severity (Foltran et al., 2011; May and

Gossage, 2011; Riley and McGee, 2005). The fetus in the first trimester is sensitive to ethanol exposure because brain cells are migrating and proliferating (Miller, 1996). Organogenesis also occurs in the first trimester making the time of exposure sensitive to specific organs. Between human gestation weeks three to sixteen are particularly sensitive because of the gastrulation stages of embryogenesis, when deficiencies in the anterior neural plate and prechordal mesoderm result in the craniofacial phenotypes associated with FAS (Sulik et al, 1984). It is thought that the most severe and consistent effects are seen when the fetus is exposed to ethanol in early gestation, but others believe PAE is more severe if exposed the entire length of gestation (Korkman et al., 1998; Wacha and Obrzut, 2007). Neuronal loss has been found in the second trimester (Barnes and Walker, 1981; Miller and Potempa, 1990) and third trimester (Bonthius and West, 1990; 1991b). Also, in the third trimester brain weight and number of neurons decrease when exposed to ethanol (Thackray and Tifft, 2001). The last two months of pregnancy are susceptible to ethanol exposure because of synaptogenesis when the brain has its final growth spurt (O'Leary, 2004; Ikonomidou et al., 2000), damaging the cerebellum, hippocampus and the prefrontal cortex (Coles et al., 1991). Embryo and specific organ development are most susceptible to the effects of teratogens during the time of rapid cell differentiation which is evident in humans and animal models.

Several genetic factors play a role in FASD severity. Genetics determine how ethanol is metabolized by the body and an individual's sensitivity to alcohol, transportation of nutrients across the placenta, and uterine blood flow. The metabolism

of alcohol varies among individuals and from one pregnancy to the next (May and Gossiger, 2011). There are genetically encoded differences in alcohol dehydrogenase. This enzyme can be less functional in the mother or the fetus could be deficient in alcohol dehydrogenase activity making it less tolerant to elevated maternal alcohol levels (McCarver, 2001; Thackray and Tifft, 2001; Ismail et al., 2010). If alcohol is metabolized less efficiently from the liver then higher peak blood alcohol concentrations result from drinking alcohol. In South Africa, women with FAS children produced higher blood alcohol concentrations more quickly than women who drank during pregnancy but whose children were not diagnosed with FASD. It was found that the women with FAS children did not have the more protective genetic variant of alcohol dehydrogenase (Khaole et al., 2004).

Nutritional deficiencies are common in mothers with FAS. These mother's diets are low in riboflavin, calcium, and omega-3 fatty acids (May et al., 2004). Vitamin deficiencies in alcoholics are attributed to decrease intake and absorption, and changes in metabolism. Although mechanisms are not completely understood, ethanol diminishes the bioavailability of riboflavin from the GI tract (Pinto et al., 1987). Riboflavin deficiency during gestation leads to fetal resorption (Kalter and Warkany, 1983) or malformations such as cleft lip and palate (Faron et al., 2001). Calcium is important in regulating neuronal membrane excitability and neurotransmitter release (West et al., 1994), and for intracellular calcium concentrations. An increase or decrease in intracellular calcium concentrations could lead to neuronal dysplasia (Kater et al., 1988). Omega-3 fatty acids are required in synapse membranes for

synaptogenesis, and in the response of injury to the nervous system (Simopoulos, 1991). The brains of omega-3 deficient rats were found to be more susceptible to environmental toxins and alcohol, suggesting omega-3 fatty acids influence brain development (Bourre et al. 1989). Chronic alcohol consumption has been shown to interfere with ingestion, absorption and utilization of nutrients (Thomson and Pratt, 1992), but adequate nutrition is crucial for fetal brain development. If maternal nutrition is inadequate, then nutritional distribution to the fetus will be affected.

Low socioeconomic status is a risk factor for FASD. Poor women who drink heavily during pregnancy generally use other drugs in addition to alcohol, and are heavy smokers and sometimes depressed (May and Gossage, 2011). These are risk factors for FASD because these are unstable households where the mother is often unknowing of the risk factors of their behavior while they are pregnant.

With so many factors influencing whether or not a woman drinks during pregnancy and how the ethanol reaches the fetus, it is no surprise that there is such a spectrum of outcomes, resulting in the fact that not all individuals exposed to alcohol prenatally express the same physical, behavioral and mental outcomes, or present with the facial anomalies for FAS diagnosis.

Central Nervous System

Brain anatomy

The developing brain continuously increases the degree of vascularization throughout gestation making it susceptible to the teratogenic effects of alcohol (Chang

et al., 2003). Central Nervous System dysfunction is associated with the most severe birth defects caused from prenatal alcohol exposure (PAE). The brain is highly susceptible to abnormalities from ethanol exposure during development, which can range from microencephaly to microscopic changes in neurons (Ferrer and Galofré, 1987). The brain develops in the initial 3-6 weeks (first trimester) and continues to mature in the second and third trimesters of pregnancy, so it is thought the CNS is affected throughout pregnancy, but with emphasis on the first and last trimesters during initial brain development and the last growth spurt (O'Leary, 2004).

Jones and Smith (1973) first observed an overall decrease in the size of the brain in FAS offspring in case studies of newborns. Many classical findings of FAS brain abnormalities had to wait for autopsy reports, even though FAS is not usually fatal so there are only a few cases autopsied (West et al., 1994). Now the availability of Magnetic Resonance Imaging (MRI) has made the neuroanatomical effects of prenatal alcohol exposure more readily researched. In a MRI study with 33 FASD diagnosed participants, Zhou et al. (2011) found a 10.6% reduction in brain volume, and a 5.3% reduction in overall cortical thickness compared to controls. The overall size decrease in the brain is due to decreases in specific brain regions, for example the basal ganglia, corpus callosum, and hippocampus (Mattson et al. 2001).

The corpus callosum is very sensitive to the teratogenic effects of alcohol. This structure is the major connection between the two brain hemispheres and is composed of 200 to 800 million nerve fibers (Roebuck, et al., 1998; 1999). The corpus callosum can have one of the most significant changes from FAS, from a thin corpus callosum

all the way to complete agenesis (Riley and McGee, 2005). Riley and coworkers (1995) found a decreased brain size that resulted from the midsagittal section of the corpus callosum reduced by 14% in FAS children and adolescents compared to control individuals. Patients with complete agenesis of the corpus callosum usually have ventricular abnormalities as well (Johnson et al., 1996) with the lateral ventricles further apart, and the third ventricle wider and higher placed. The affected brain regions are reported to not only have decreased size and volume, but a change in location, and a decrease in fiber tract number and connectivity (Mann et al., 2001; Nuñez et al., 2011; Riley and McGee, 2005; Wacha and Obrzut, 2007), and these reductions are not uniform across the structure (Davis et al., 2011). Animal MRI scans showed consistencies in the same brain areas being affected as in human alcoholics and children diagnosed with FASD. O'Leary-Moore et al. (2011) used MRI scans as early as gestational day 7 in the mouse to show dysmorphology in the corpus callosum, from thinning to complete absence.

The basal ganglia are a collection of nuclei considered part of the motor system, which does not have a role in cognitive functioning. The caudate nucleus within the basal ganglia is associated with learning, mental flexibility and behavioral inhibition (Nunez et al., 2011). Magnetic resonance imaging revealed children with FAS had a reduced basal ganglia caudate and lenticular nuclei regions even when the reduced brain size was controlled for (Mattson et al., 1996a). And in a prenatal alcohol exposure Sprague-Dawley rat model, Mattson et al. (1994) also found the area and ratio in the basal ganglia caudate and putamen nuclei regions to be reduced.

Intrauterine exposure to ethanol can result in abnormal development and function of the hippocampus (Berman and Hannigan, 2000; Davis et al., 2011). The hippocampus plays an important role in memory formation and function, changing short term to long-term memories (Mattson et al., 2001) especially in explicit and relational memory (Squire, 1992). Ethanol exposure *in utero* affects functional deficits in the hippocampus caused by neurochemical abnormalities during development (Sutherland et al., 1997). In the human 2nd trimester equivalence, fetuses of Sprague-Dawley rats given ethanol during pregnancy showed abnormal hippocampal mossy fiber distribution, some to the extent of hydrocephaly (Fukui and Sakata-Haga, 2009). Five percent ethanol throughout gestation was enough to produce a deficit in synaptic plasticity in the hippocampal dentate gyrus in adult Sprague-Dawley offspring, which may lead to a range of cognitive and behavioral abnormalities if cortical connectivity cannot be modified by early experiences (Sutherland et al., 1997).

Fetal ethanol exposure affects the fetus directly, as it can readily cross the placenta and blood brain barriers, but it may also affect brain function indirectly through secondary effects. Alcohol can constrict the umbilical vessels causing hypoxia (Mukherjee and Hodgen, 1982) so that an insufficient amount of oxygen reaches the fetus. Cerebellar purkinje cells and hippocampus pyramidal cells are susceptible to hypoxia (Brierley and Graham, 1984), and Kelly et al. (1990) found that cerebellum and hippocampus capillary diameter also decreased due to fetal alcohol exposure, altering microvasculature development in these areas. Hypoxia may also change the

acid-base balance in the fetus, known as fetal acidosis, which is associated with brain activity alterations (Mann et al., 1975).

Hypothalamic-Pituitary-Adrenal Axis

It is known that ethanol consumption changes the adult endocrine function and stimulates adrenal activity, and may be dose related by acting as a stressor. Chronic alcohol models have shown ethanol to eliminate normal diurnal variation of corticosterone and to result in enlarged adrenal glands (Kakihana et al., 1968; Ellis, 1966). Increased adrenal gland weights were found in Sprague-Dawley rat dams (Weinberg and Bezio, 1987). The dams given ethanol during gestation had greater relative adrenal weights at 16 days of gestation and both absolute and relative increased adrenal weights at day 21. Not only does the dam typically have increased adrenal weights but the offspring did as well. Kakihana and coworkers (1980) found Sprague-Dawley pups prenatally treated with ethanol had greater relative adrenal weights compared to control or sucrose treated pups. Enlarged fetal adrenal glands provided evidence that maternal ethanol consumption activated the fetal adrenal glands though the mechanisms are unknown.

The response of ethanol on activation of the hypothalamic-pituitary-adrenal (HPA) axis has been shown in *in vivo* and *in vitro* studies. In perfused adult male Wistar rat adrenal glands, Cobb et al. (1981) found ethanol and acetaldehyde increased production and secretion of corticosterone, and Kinoshita et al. (2001) found increased plasma corticosterone concentrations within 30 minute of ethanol administration to

adult male Sprague-Dawley rats, supporting the hypothesis that ethanol directly stimulates the adrenal cortex of the individual receiving ethanol. Basal glucocorticoid levels are also increased in response to stressors in pregnant ethanol consuming females, suggesting adrenocortical hypersecretion that progresses throughout gestation. By increasing basal glucocorticoid levels, the set point of the homeostatic feedback mechanism that regulates pituitary-adrenal function is increased (Levine and Mullins, 1966) and the HPA axis is hyperresponsive to stressors (Weinberg and Bezio, 1987). The ethanol increase in plasma corticosterone concentrations was dependent on animal strain, dose, time between injection and blood collection, and the nature of stressor (Kakihana et al., 1968; Ellis, 1966; Weinberg, 1992).

Maternal alcohol use changes her endocrine function directly at the glands and at the hypothalamus and pituitary as stated above, however it also can disturb the interactions between the maternal and fetal hormonal systems, indirectly affecting the development of fetal metabolic, physiologic, and endocrine functions (Zhang et al., 2005; Weinberg and Bezio, 1987). Prenatal ethanol exposure changes the HPA axis which results in hyperresponsiveness, poor coping adaptations (Weinberg, 1988; Bilitzke and Church, 1992) and deficits in HPA recovery after repeated exposures to stress (Weinberg et al., 1996). Prenatal ethanol exposure and chronic mild stress has long-term consequences on the sensitivity to stressors, leading to an increase in responsiveness to stressor in adulthood such has hyperactivity, altered social interactions, and anxiety-like behaviors (Hellemans et al., 2008; Hellemans et al., 2010).

HPA responsiveness in offspring prenatally exposed to ethanol may not be specific to ethanol, but from ethanol increasing HPA responsiveness to stress (Taylor et al., 1982a). Plasma and brain corticosterone concentrations are greater at birth in prenatally ethanol exposed Sprague-Dawley rats (Kakihana et al., 1980) and female B6SJL/F1 mice (Allan et al., 2003), and can persist until three days after birth (Taylor et al., 1982b). However, differences in HPA responsiveness are usually only found under challenged and stressed conditions and not under basal conditions because PAE increases adrenocortical sensitivity to chronic stress. Prenatally ethanol exposed animals have shown a prolonged increase in cortisol and ACTH concentrations during and after different stressors were applied (Weinberg, 1988; Weinberg, 1992). However, stress-induced HPA activity can influence HPA responsiveness in a selective manner. Taylor et al. (1982a) observed increased corticosterone responses only to cardiac puncture and noise and shake stressors, and not to novelty, environmental or metabolic stressors in PAE adult rats. The stressors that elicited increased corticosterone concentrations were composed of restraint, pain and fear by nature. However, neonatal PAE rats showed reduced adrenocortical responses to drug (ethanol and morphine) challenges (Taylor et al., 1986). It may not be until weaning age or puberty that prenatally ethanol exposed animals exhibit hyperresponsiveness to stressors that continue until adulthood.

Ethanol may not directly activate the adrenal cortex but rather stimulate adrenocorticotropic hormone (ACTH) secretion via the hypothalamus and pituitary. Ellis (1966) observed that in hypophysectomized and pentobarbital-morphine injected

Sprague-Dawley rats, ethanol failed to elicit an increase in adrenocortical response as seen in control rats. A possible mechanism of action is a deficit in feedback regulation of the HPA axis that may result in hyperresponsiveness to stressors, due more so to the inability to terminate the stress response and not a prolonged sensitivity to a stressor (Sapolsky et al., 1984). If circulating cortisol concentrations were higher than the set point, ACTH and adrenal output are decreased, and if cortisol concentrations were below the set point then ACTH is released. When stressors, such as ethanol, were applied during critical periods of development, cortisol concentrations varied, altering the set point for the feedback mechanism (Levine and Mullins, 1966). The hippocampus has been suggested to have a role in negative feedback actions of glucocorticoids because it is one of the most vulnerable areas of the brain to ethanol's teratogenic effects (Sapolsky et al., 1984). Deficits in hippocampal development may decrease the number of hippocampal glucocorticoid receptors, leading to hypersecretion of glucocorticoids. Increased mineralocorticoid and glucocorticoid receptor mRNA concentrations in the hippocampus have been observed (Glavis et al., 2007), but Weinberg (1992) found no differences in maximal binding or binding affinity in type I or type II hippocampal glucocorticoid receptors. The increased HPA response seen in prenatally ethanol exposed offspring is not from altered adrenal sensitivity to ACTH augmenting corticosterone responses, but rather from maternal ethanol consumption exerting long-term effects on the fetus' hypothalamus and pituitary that mediate HPA activation (Taylor et al., 1982a), and is more likely a

reflection of ethanol stimulatory action on the fetal pituitary-adrenal system than on the maternal HPA axis (Taylor et al., 1982b; Kakihana et al., 1980).

Immunology

Immune system regulation is altered by alcohol abuse and often leads to immunodeficiency and autoimmunity (Cook, 1998). Alcoholics are usually immune-compromised and are susceptible to infections and have decreased ability to fight these infections (Roselle et al., 1993) because the innate and adaptive functions of the immune system are impaired or inappropriate to the pathogen (Szabo, 1999).

Innate immunity elicits the immediate activation of phagocytic cells to locate, ingest and kill pathogens through oxidative burst. Exposure to ethanol both acutely and chronically impairs phagocytic potential and the ability of these cells to present the antigen to the adaptive immune system (Jerrells and Weinberg, 1998). Decreased levels and expression of neutrophils and macrophages have been found in chronic and acute ethanol exposure, along with impaired phagocytic activity (Mufti et al., 1988), while liver macrophages (Kupffer cells) only decrease during chronic alcoholism (Goral et al., 2008). During alcohol exposure, chemotaxis of neutrophils were increased in rats (Bautista, 1997) but decreased in humans (Patel et al., 1996). This difference shows more research is needed in how the immune system is impaired and the role chemokines play across species. It was suggested the murine model may not be a good immune model for the human. The pig is 80% similar to humans in their

immunity and could make a good model for the affects of alcohol on immunity (Schook et al., 20050.

Phagocytic cells generate active oxygen radicals, superoxide anion, nitric oxide and hydrogen peroxide to kill pathogens through oxidative burst. Ethanol can inhibit production of these radicals which decreases secretion and impairs the innate immune response (D'Souza et al., 1996). Innate immunity also responds to pathogens by releasing pro-inflammatory cytokines as a first line of defense and to activate the adaptive immune response. Acute alcohol decreased the inflammatory response while chronic exposure increases it (Goral et al., 2008; Szabo and Mandrekar, 2009). Prolonged inflammatory responses lead to other diseases often seen in alcoholics such as, hepatitis, pancreatitis, and septic shock (O'Brien et al., 2007) and have a role in cancer etiology (Lawrence et al., 2007).

The adaptive immune response is mediated by lymphocytes and on first encounter requires activation by the innate immune mechanisms (antigen presenting cells and pro-inflammatory cytokines) but is immediately activated on subsequent encounters of the same pathogen. Humoral immunity (B-cell antibody mediated on extracellular cells) and cellular immunity (T-cell mediated on intracellular cells) are the two effector mechanisms that exist to eliminate pathogens in adaptive immunity. Alcohol can alter the production of both T and B cell lymphocytes. T-cell numbers are reduced and non-specific activation of B-cells can occur in alcoholics (Dunne, 1989). The adaptive immune system is very specific. Without this specificity, a person could die from septic shock because all the lymphocytes would be activated. Non-specific B-

cell activation would be detrimental for an individual. Mufti et al. (1988) found an increase in T-Helper cells in the splenocytes of rats to compensate for the overall loss in the T-cell population throughout the body. If T-Helper 1 (help macrophages kill pathogen by increasing phagocytosis) and T-Helper 2 (help B-cell make antibodies) proportions are skewed in one direction, immunological disease may result. Chronic alcoholics have excess TH2 function, which is a proposed reason for their immune abnormalities (Cook, 1998).

Not only does alcoholism immediately affect the maternal immune system, but it has been found that PAE also plays a role in offspring immunity. Johnson et al. (1981) found 13 FASD patients had an increase in bacterial infection such as, meningitis, pneumonia, and sepsis. And Grossmann et al. (1993) observed 22% mortality due to infectious disease in the offspring of binge drinking *Macaca nemestrina* nonhuman primates. Defects in cellular immunity can lead to viral and fungal infections, and humoral immunity deficits can predispose individuals to sepsis, meningitis, and pneumonia due to decreased lymphocyte proliferation (Johnson et al., 1981; Zhang et al., 2005).

Deficits in adaptive immunity in prenatal ethanol exposed individuals have shown low cell counts of T-cells, decreased lymphocyte proliferation, and decreased immunoglobulins in humoral immunity (Gottesfeld and Abel, 1991; Zhang et al., 2005), and B cell maturation can be delayed in the liver (Biber et al., 1998). Johnson et al. (1981) examined the cell-mediated immune and the humoral immunity of FAS children. They found measures of cell-mediated immunity, absolute lymphocyte

counts, and mitogen-induced stimulation, to be depressed except in skin delayed hypersensitivity. Humoral immunity showed decreased absolute B lymphocytes, and various abnormalities in IgG, IgA, and IgM concentrations in FAS children but no abnormal quantitative immunoglobulin levels were observed in control children.

The thymus is a location of lymphocyte production. A single high dose of ethanol reduced thymic weight and altered the ratio of the cortex and medulla (Budec et al., 1992). Thymus deficits are seen in individuals exposed to ethanol prenatally as well. C57BL/6 mice prenatally exposed to a 25% diet of ethanol had a smaller thymus, decreased expression of thymocyte differentiation antigens, and no delineation between the cortex and medulla of the thymus (Ewald and Walden, 1988). These data showed that fetal alcohol exposure led to thymus immaturity and an immature thymus may not be able to elicit an efficient T-cell response to a pathogen. Mice fetuses at 18 and 19 days of gestation from "chronic alcoholic" dams had one-third to less than one-tenth the number of thymocytes in the thymus and had a low proliferative response to mitogen Concanavalin A compared to control fetuses (Ewald and Frost, 1987). Weinberg and Jerrells (1991) also found a decrease in thymocyte number and a decrease in lymphocyte response to a mitogen in PAE male Sprague-Dawley rats because they were unable to utilize exogenous interleukin (IL) 2. DiGeorge Syndrome is the complete absence of the thymus, sometimes found in prenatally ethanol exposed children (Barrett et al., 1981; Ammann et al., 1982), and the characteristics of DiGeorge Syndrome are very similar to the facial and clinical features of FAS (Johnson et al., 1981).

Early studies have found lower cell counts of eosinophils and neutrophils (Gottesfeld and Abel, 1991; Zhang et al., 2005), and decreased phagocytosis in macrophages (Gauthier et al., 2005; Ping et al., 2007). However, sometimes deficits in innate immunity are not seen in animal models (Zhang et al., 2005). Grossmann and coworkers (1993) did not find differences in white blood cell counts, monocyte numbers, or monocyte phagocytic function, nor did Johnson et al. (1981) observe differences in neutrophil function in FAS children, though they did find an increase in eosinophil numbers.

There is sufficient evidence that alcohol affects the immune system function both in alcoholism and fetal alcohol exposure. However, it is unknown if alcohol itself or its metabolites are directly responsible for the alterations in the innate and adaptive immune responses, or if alcohol is indirectly acting on nutrition and hormones to suppress immunity.

Behavioral implications

Children with FASD do not present with the facial features characteristic of FAS, so the cognitive-behavioral deficits are routinely used to help with diagnosis. The behavioral phenotype of FASD has been mapped to include academic difficulty, emotional dysfunction and social dysfunction (Kodituwakku, 2007). FASD children were characteristically described similarly among their caretakers; even before there were many readily available writings about the disorder, leading Streissguth et al. (1998) to compile a list of descriptors making a behavioral scale including but not

limited to: mood swings, poor judgment, overstimulation, poor manners, not a team player, does not complete tasks, unaware of consequences, and overreactivity.

The brain structure and connectivity changes caused by prenatal ethanol exposure have direct consequences on the behavior of offspring. The cerebellum is responsible for motor functions such as balance and coordination (Davis et al., 2011). Meyer et al. (1990) exposed rat pups to ethanol neonatally during a brain growth spurt, and found an abnormal gait of shortened stride length and increased angle of placement of the hindfeet. Attention deficits, intellectual functioning, learning, verbal memory and psychosocial functioning have been linked to abnormalities in the corpus callosum (Mattson et al., 2001). The corpus callosum allows the two hemispheres of the brain to communicate. The basal ganglia, more specifically the caudate nucleus, is involved in cognitive function and has neural connection to the frontal lobes which mediate higher cognitive and executive functions (Mattson et al., 2001). When the hippocampus was damaged, the ability to store new memories was lost and spatial navigation learning was altered (Wilson and Cudd, 2011).

Neurobehavioral deficits, including impairments in learning and memory, attention, reaction time, motor skills, social and adaptive behaviors, hyperactivity and developmental delays are now commonly identified in children with FASD and verified in animal models (Wilson and Cudd, 2011). They can be an important tool for diagnosing FASD when the structural abnormalities are not present. However, there is much variability in the cognitive dysfunction profile of individuals with different levels of ethanol exposure *in utero*. The behavioral and cognitive characteristics of

FASD help with diagnosis, but no single behavioral phenotype has been identified for FASD making diagnosis at the clinical level questionable (Astley et al., 2009).

Socialization

Social impairments such as poor judgment, impulsivity and inability to understand consequences (Davis et al., 2011) are seen in FASD individuals. Global functioning characterization tools have been used to give insight into social functioning in FASD individuals. These include the Child Behavior Checklist (CBCL) which measures social competence and problem behaviors, and the Vineland Adaptive Behavior Scales (VABS), which measures adaptive functioning (Kully-Martens et al., 2012).

Child Behavior Checklists and Teacher Rating Forms questionnaires have shown social problems to be one of the most common profiles of FASD individuals over subsequent interviews (Steinhausen and Spohr, 1998). The CBCL measures three competence areas in activities, social settings and school settings, and eight problem scales: withdrawn, somatic complaints, anxious/depressed, social problems, thought problems, attention problems, delinquent behavior and aggressive behavior (Mattson and Riley, 2000). As early as infancy, children can show conflicting behavior towards separation from their mother or caretaker and are highly irritable (Kelly and Streissguth., 2000). If attachment issues start at infancy when the baby is reliant on the caretaker, this can be a possible predictor of later behavioral problems. Mattson and Riley (2000) found PAE children had elevated internalizing problems (withdrawn,

anxiety/depression) but only social, attention and aggressive behaviors were significantly different from matched control children.

The Vineland Adaptive Behavior Scales rate children in communication skills, daily living skills, motor skills, and socialization. Whaley et al. (2001) found deficits in adaptive function across all three domains as expected in 66 children exposed to different levels of prenatal alcohol. Thomas and coworkers (1998) analyzed three subdomains of social skills, interpersonal relationships, coping skills and use of play/leisure time, and found that children with FAS were significantly impaired in all areas of social skills compared to children with similar IQs and normal controls. They also concluded that age negatively impacted social ability. Therefore, as FAS children get older they lag further behind peers in social skills.

The social interaction test has been validated as a test of anxiety and social behavior in animal models. Prenatal ethanol treated Sprague-Dawley male rats spent less time performing social and non-social behaviors in the first five minutes of a social interaction test, but in the last five minutes performed more non-social behaviors (Hellemans et al., 2010).

Social problems can escalate with age. At adolescence when the social demands are more complex, social deficits in FASD children become more prominent (Kodituwakku, 2007). Adolescent and adult individuals with FAS, on average, displayed social skills at a six-year-old level (Whaley et al., 2001). Even with normal IQ scores, FASD individuals may still be at greater risk for behavioral problems and psychiatric disorders because of the adaptive problems (Thackray and Tift, 2001).

Without proper social skills needed to establish and maintain relationships, individuals with FAS may exhibit behavioral problems such as aggression, hyperactivity, poor control, and delinquent behavior (Kupersmidt et al., 1990; Streissguth et al., 1996). The increased risk of peer rejection can contribute to aggression, anxiety and depression, antisocial behavior, and addictive behaviors characteristic of FASD (Kully-Martens et al., 2012).

Hyperactivity

Many children diagnosed with FAS are considered inattentive and hyperactive. They are often compared to children with attention-deficit/hyperactivity disorder (ADHD) (Riley and McGee, 2005), and are deficient in maintaining attention and inhibiting impulsive responses (Mattson et al., 2011). Even though psychiatric evaluations show FASD individuals are diagnosed with ADHD (Fryer et al., 2007; Steinhausen and Spohr, 1998), it is thought that FAS and ADHD children may have attention deficits in different areas. Coles et al. (1997) found children with FASD and ADHD had similar intellect. When looking at the dimensions of attention though, children diagnosed with ADHD had difficulties in sustaining and focusing tasks, while FASD children had more difficulty with tasks involving encoding and shifting, or the ability to learn and manipulate new material in working memory while processing into long term memory, and flexibility in response to new information, respectively (Coles, 2001; Coles et al., 1997).

Several apparati can be used to assess activity in animal models, such as open field tests and elevated plus mazes. Ledig et al. (1990) found offspring of Wistar strain female rats acclimatized to alcohol for 5 weeks before mating crossed more squares and reared more than control rats. Hellemans et al. (2010) found pair-fed and prenatally ethanol exposed Sprague-Dawley male rats were significantly more active than control rats. However, female rats, regardless of treatment, were more active than males, the PAE mice in the C57BL/6 strain were also more active than controls (Becker and Randall, 1989). Increase in locomotion in the rat model gives support to the claim that hyperactivity is a commonly reported behavioral symptom of FASD. An increased locomotor activity can be associated with increased anxiety and fear (Carola et al., 2002), which is also characteristic of FASD. In contrast, PAE has been found to have no effect on locomotor activity in Sprague-Dawley rats (Brocardo et al., 2012) or Wistar rats (Dursun et al., 2006), while reduced activity was found in C57BL/6 mice (Kleiber et al., 2011) and in Sprague-Dawley rats (Hellemans et al., 2008).

Adolescent prenatally alcohol exposed and/or prenatally stressed Rhesus monkeys' behaviors were assessed while performing the Wisconsin General Test Apparatus learning and memory tasks. Across multiple acquisition tasks monkeys exposed to both alcohol and stress prenatally displayed more stereotypies and were more active, and alcohol exposed monkeys were more irritable. Schneider et al. (2001) associated the monkeys' slower acquisition learning to their behavior irritability, activity, impulsivity and increase in stereotypies. Activity and impulsivity are characteristics of FASD and can be linked to the inability to perform well on other

non-related tasks. However, Nulman et al. (2004) found children from mothers who binge drank during pregnancy performed high on the distractibility scale (non-distractible) in a temperament test. Non-distractibility in these children reflected more of an inability to disengage from an ongoing task.

Anxiety and Fear

The teratogenic effects of ethanol can affect offspring coping ability resulting in high anxiety and poor stress adaptation. It is well documented and discussed above that prenatal ethanol exposure increases hyperresponsiveness to stressors (Weinberg, 1988; Weinberg, 1992). The hyperactivity and dysregulation of the HPA axis can cause individuals with FASD to have greater rates of depression and anxiety disorders (Hellemans et al., 2008)

The forced swim test measures the duration of immobility in a cylinder of water. The amount of immobility directly reflects the level of stress or fear so that the more mobile with increased struggling and escaping, the more fearful the animal. Bilitzke and Church (1992) found male Long-Evans hooded rats prenatally exposed to ethanol were less immobile than control rats, suggesting they were more fearful or were unable to calm down once excited. However, typical results from forced swim tests show an increase in immobility. Depressant effects of alcohol are seen in prenatally ethanol exposed Sprague-Dawley rats (Hellemans et al., 2010; Brocardo et al., 2012) and Wistar rats (Carneiro et al., 2005) by an increase in immobility compared to controls. Increased immobility reflects a state of "behavioral despair,"

because the animal gives up and has learned escape is impossible (Porsolt et al., 1978; Carneiro et al., 2005) or this strategy could be an energy conserving coping strategy (Borsini and Meli, 1988).

The elevated plus maze can also be used as a measure of anxiety. The apparatus consists of two open arms and two closed arms connected by a central platform that is elevated above the floor. There is a natural aversion for rodents to be in the open arms, and the level of anxiety can be measured by the relative amount of time spent in the open arms compared to closed arms. Rats prenatally exposed to ethanol have higher levels of anxiety and spend less time in the open arms (Brocardo et al., 2012; Dursun et al., 2006). However, Carneiro and coworkers (2005) found rats prenatally exposed to both low (0.5 g/kg) and moderate (4 g/kg) doses of ethanol entered and spent more time in open arms compared to closed arms, suggesting that prenatal ethanol exposure had an anxiolytic effect on rats. Osborn et al. (1998) observed a sex effect in fear factor of the elevated plus maze. Male rats prenatally exposed to ethanol showed lower levels of fear by spending more time in open arms, but females tended to spend less time in open arms indicating higher levels of fear. There was no difference in activity within the open arms, but PAE males and females were more active in the closed arms. There can be differences in the behavioral response to anxiety tests based on strain. Carola and coworkers (2002) observed that C57BL/6 mice were explorative and active in the closed arms of the elevated plus maze, but that BALB/c mice were inactive, less explorative and stayed in the central platform, and had high corticosterone levels.

Novelty and Addictive Personalities

The devastating consequences of FASD continue throughout adolescence and adulthood as personality characteristics such as poor judgment, inability to understand consequences, impulsivity and sensation/novelty seeking (Mattson et al., 1999; Davis et al., 2011). Although individuals with FASD tend to be more anxious and fearful, they are more willing to approach novel objects or strangers. For animal models the open field is also an assessment of novelty seeking behavior. Ledig et al. (1990) found an increase in the time treated rats spent in a novel compartment more than a familiar compartment, showing less avoidance towards novelty, while control rats showed no preference for either compartment. Prenatally ethanol exposed mice spent more time in the center of an open field apparatus in the presence of a novel object suggesting greater exploratory activity than saccharin exposed controls (Allan et al., 2003) though there were no difference in number of entries into the center regardless if the novel object was present or not.

During adolescence, FAS teenagers are impulsive and use poor judgment (Thackray and Tifft, 2001) without understanding the consequences, these poor decisions could be life altering. Even children of non-alcoholic mothers who were binge-drinkers exhibited social disinhibition. These children were described as more than normally willing to seek out strangers and novel surroundings, were overly friendly, more adaptable to new situations, and less fearful of change than controls (Nulman et al., 2004). Prenatally ethanol exposed individuals are two-fold more likely to have passive-aggressive and antisocial personality traits and develop substance abuse disorders (Fryer et al., 2007). Problems with impulse control and high levels of

aggression at an early age increase the likelihood of alcohol abuse in adolescence and early adulthood (Gunzerath et al., 2011).

Prenatal ethanol exposure increases the offspring's postnatal responsiveness to ethanol, even programming the reward circuitry of the brain (Foltran et al., 2011). The exact mechanisms that reinforce ethanol preference after prenatal exposure are unknown, although the chemosensory system is believed to be involved. It has been suggested that *in utero* exposure to the taste of ethanol allows for associative learning, so that when the individual first experiences ethanol during adolescence it pharmacologically reinforces the effects of the alcohol (Pautassi et al., 2012). Adolescence is the period in an individual's life when alcohol consumption is accelerated (Gunzerath et al., 2011), and the likelihood of abusing alcohol is great. Animal research has shown prenatally exposed rats were sensitized to the appetitive effects of ethanol at an early age, but that preference may not still be there at adulthood (Nizhnikov et al., 2006; Pautassi et al., 2012; Youngentob et al., 2007a; Youngentob et al., 2007b). If the FASD individual could avoid situations that emphasize intake seeking behaviors throughout adolescence, then the reward reinforcement of ethanol may not be there when they drink as an adult and could potentially decrease their chances of becoming an alcoholic.

Memory

Prenatal exposure to ethanol can affect learning and memory, including verbal, nonverbal and spatial learning. hildren with FASD learned fewer words and had

greater difficulty recalling words (Mattson et al., 1996b; Mattson and Roebuck, 2002). However, it was suggested that these deficits were in the acquisition of the material and not necessarily in the ability to remember the information over time (Mattson et al., 2001). Difficulty in both free recall and recognition recall have been observed (Crocker et al., 2011; Mattson et al., 1996b), or were observed in free recall only (Kodituwakku, 2007; Mattson and Riley, 1999). These deficits observed provide support for impairment in encoding verbal information and response inhibition capabilities characteristic of FASD (Mattson et al., 1996b).

Human studies can use intellectual and neuropsychological measures to provide insight into cognitive deficits. There are multiple tests available to assess different functions across different age groups, and each test is often chosen based on its sensitivity to the deficits frequently observed in FASD or to specific brain region functions. Neuropsychological tests assessing attention, memory/learning, and executive functioning in non-mentally retarded, average IQ and below average IQ individuals with FAS were chosen by Kerns et al. (1997) because they depended on the integrity of the frontal lobe. The average IQ FAS group performed lower than expected on measures of attention, demonstrated a lower level of learning in verbal memory but good retention of what was learned when visually observed. Individuals were able to retrieve information once it was acquired but had difficulty with the initial acquisition and encoding of new material, and required more repetition. The below average IQ FASD group displayed more severe neuropsychological deficits than the average IQ FASD group. They had average basic sustained attention but had

more difficulty on complex tasks. In verbal memory, they had below average recall and a shallow learning slope with less ability to organize the material trying to learn. This suggests they had a shallow level of encoding and difficulty using effective learning strategies, but did benefit from cuing strategies. Understanding the differences in cognitive deficits could help facilitate learning in children with FAS as it is not their lowered IQ that explains their cognitive deficits.

Behavioral disturbances observed in FASD individuals such as impulsivity and poor judgment is consistent with deficits in executive functioning. Executive functioning deficits impeded daily functioning and activities, limited independence and hindered positive social interactions (Mattson et al., 1999). Manipulation of information and goal management in working memory, which is the ability to use information in short-term memory, were impaired (Kodituwakku et al., 1995). Other areas of executive function investigated include planning, cognitive flexibility, selective inhibition, and concept formation and reasoning. Mattson and coworkers (1999) gave children diagnosed with FAS and those exposed to alcohol prenatally a series of tests measuring the different domains of executive function compared to controls. Deficits in all areas of executive functioning were observed in alcohol exposed groups and in most cases did not differ from each other but did from controls.

Malisza et al. (2005) evaluated working and strategic memory in children and adults with FASD using Self Ordered Pointing Task, vigilance using the Continuous Performance Task, and executive function in the Wisconsin Card Sorting Task, along with spatial memory in a series of n-back tasks while participants were in a MRI.

Briefly, the methods for the series of n-back tests were first a simple Task (n=0), where study participants indicated with a button the spatial location of a colored circle, after a brief delay blank circles were presented in a Blank Task (n=1), or one (One-back task; n = 1) or two (Two-back task; n =2) stimuli were presented. Overall FASD individuals in the Self-ordered Pointing Task committed more errors and children had a harder time with the task than adults, and the Continuous Performance Test revealed that the FASD participants performed similarly to control children except they took significantly longer to make a correct response compared to control participants. In the n-back tasks, FASD individuals made fewer correct responses, took longer to make a correct response, and had higher rates of incorrect and non-responding answers than controls. Functional MRI scans the n-back tasks showed greater activity in the inferior and middle frontal cortex than controls, suggesting prefrontal areas involved in executive functioning were improperly working.

Astley et al. (2009) also investigated MRI and fMRI neuroactivation across four medically diagnosed groups under FASD: FAS, Static Encephalopathy (SE/AE) and Neurobehavioral Disorder, Alcohol Exposed (ND/AE) and control groups. They found that the n-back working memory task decreased significantly and incrementally progressing across the four groups. MRI data revealed a smaller frontal lobe only in the FAS group, and the caudate was smaller in the FAS and SE/AE groups, with neurostructural abnormalities also being observed in the ND/AE group. Brain abnormality severity increased as individuals progressed in CNS dysfunction (Astley et al., 2009).

Nonverbal skills rank similarly to verbal skills with lower rate of learning over multiple trials and less recall after a delay period (Mattson et al., 2011). Visual-Spatial memory is another index to measure cognitive function. Prenatal alcohol exposure impairs spatial response dependent learning as well, and is a better assessment for learning and memory in animal models. Several maze types were used for evaluating spatial learning and memory. The T maze and plus maze allow the animal to explore the maze until it makes a decision (right or left turn) and enters the goal box. The maze test is then repeated until the animal chooses the opposite goal box from its first decision. The radial arm maze has a central octagonal area with various arms coming off as spokes, usually eight. In general a reward is placed in an arm, and the numbers of correct and incorrect choices to find the reward are recorded. The Morris water maze is a commonly used measure of spatial memory in rodent models. The animal swims to a hidden escape platform submerged in a pool. This maze uses distal and proximal visual cues to map the task. And Hebb-Williams mazes start with an open arena with no internal borders to locate the goal. In the subsequent trials, internal borders are inserted but the goal remains in the same location and subsequent trials use different maze configurations. All of these spatial learning and memory apparatuses assess reference memory, remembering the goal location, and working memory, learning the location of the goal to avoid errors.

Hippocampal dysfunction caused by PAE is commonly assessed in various adaptations of the above mentioned spatial learning and memory tasks. The general hypotheses from these tasks are an increase in number of errors and latency to

complete the task in prenatal ethanol exposed animals. In the T maze, prenatally alcohol exposed Long-Evans rats required more trials in reversing to the new goal (opposite of first trial of free choice) (Riley et al., 1979) and made more reference and working memory errors (Zimmerberg et al., 1991). However when observing different strains of mice (C57BL/6J, BALB/cJ, and DBA/2J) in a radial arm maze, Sluyter et al. (2005) only found a strain difference and not a prenatal alcohol exposure difference. In Sprague-Dawley rats, only 50% of rats from dams given a 35% ethanol diet during gestation were able to complete the radial arm maze and required twice as many trials to meet criteria as control rats. It took both the 35% and 17% prenatal ethanol diet treatments less time to complete the maze per trial, irrespective of the number of errors, compared to their pair-fed controls. However, Reyes et al. (1989) concluded that the prenatal alcohol exposed rats simply moved more quickly between arms with less purpose in early trials, which is consistent with evidence of FASD increasing activity.

In a radial arm maze that assessed both visual cues and spatial contingency, prenatally exposed rats made more errors when switching from cued to spatial condition (Berman and Hannigan, 2000), which is consistent with findings in the Morris water maze. The Morris water maze uses distal cues (hidden platform) to assess place learning and proximal cues (visible platform) to assess cued-navigation. When measuring distal cue spatial navigation, PAE treatments took longer and traveled greater distances to find the hidden platform, but when assessing proximal cued spatial navigation there were no longer any differences between treatments in

Sprague-Dawley rats (Kelly et al., 1988), Long-Evans rats (Cronise et al., 2001), C57B16/J mice (Endres et al., 2005) and even in adolescent boys (Hamilton et al., 2003). There were differences in the rate ethanol treated groups learned the task compared to controls, but once the task was mastered, there no longer were differences in search strategies in the animal model. During the no-platform probe trial in Hamilton et al.'s (2003) virtual Morris water maze (VMWT) study, FAS adolescences spent more time in the other regions of the pool, and not the quadrant the platform had previously been in. They took longer and significantly greater distance to enter the correct quadrant while searching less persistently in the correct quadrant than the control group, which supports the interpretation of impaired place learning caused by hippocampal dysfunction in FAS and not from visual-motor or motivation deficits.

Pig models

Although human research has been important in identifying the spectrum of fetal alcohol exposure and new neuroimaging technologies make human studies more comprehensive, there are still many areas of alcohol and fetal alcohol research that human research cannot explore. Human studies rely on self-reporting of alcohol consumption and often alcohol isn't the only drug of abuse, which can confound the study (Wilson and Cudd, 2011). Animal models allow the researcher to control the environment while working on living organisms that would not be ethical for human subjects (Evans, 1979), though there are limitations to animal models in extrapolating the data to human medicine. When studying different periods of alcohol consumption,

getting the animal to voluntarily drink to meet Lester and Freed's (1973) alcohol model criteria can prove to be difficult. Animal models are used as an attempt to bring greater understanding to some behavior that is an analog to a human behavior at the physiological, biochemical or molecular level, with either face validity or predictive validity (Tabakoff and Hoffman, 2000). Here, we focus on the use and significance of the swine model for FASD and alcoholism.

The domestic pig model could help bridge the gap between extrapolating animal model findings to the human, as they are very similar to humans in their anatomy, physiology and behavior. The domestic pig is becoming more commonly used in biomedical research because of its availability and relatively inexpensiveness. It is more similar to the human than the rodent model and less expensive compared to nonhuman primates. The use of nonhuman primates and other companion species such as dogs have strong ethical concerns with activist groups. However, pigs are still widely seen as a production animal so there is less controversy in the public view of their use (Gieling et al., 2011), although minipigs are becoming more popular as pets.

Neuroimaging techniques are also being utilized in the animal model. The pig brain is gyrencephalic like the human brain and, therefore, similar in structure and function, which makes it a useful model in neuroscience research. Numerous imaging studies in the pig model have shown the growth (Conrad et al., 2012), and function to visual stimuli (Fang et al., 2006) and painful stimuli (Fang et al., 2005) on the brain. Complete brain atlases are available (Saikali et al., 2010; Watanabe et al., 2001), which map the regions of activation in the pig brain.

The pig is also an appropriate animal for pharmacokinetic evaluation (Gieling et al., 2011) and the perfused pig liver has approximately the same ethanol elimination from the liver as humans (Keiding et al., 1979). Most of the pig FASD research to date is from one laboratory working with Sinclair miniature pigs and is a few decades old. This group from Missouri established this breed of sows as a voluntary drinker of ethanol up to 30% w/v, one of the criteria for a good model of alcoholism (Dexter et al., 1980; Dexter et al., 1976; Tumbleson et al., 1981a; Tumbleson et al., 1981b). Dexter et al. (1976) first established that Sinclair mini-sows would voluntarily consume ethanol to the equivalent of human alcoholics and go through both intoxication and withdrawal states. However, Cloutier et al. (2006) used crossbred control and ethanol fed sows in a choice test to see if they actually favored ethanol. Both control and ethanol groups preferred the plain feed and feed with dextrose compared to feed with alcohol. However, neither group showed a bias towards a particular diet as there was no difference in time to first approach the buckets with the feed treatments. Toward the end of the test, sows found exploring the arena more rewarding than finishing the alcohol diet. These sows would eat the ethanol diet when it was its only food source, but when given the choice they avoided the highly concentrated (27%) diet.

When measuring sow reproductive performance, the minipig model resulted in characteristic FAS observations. In one study of second generation drinking sows, litter size and birth weight were decreased, and percent of stillbirths increased. The offspring showed characteristic anomalies of FAS such as microcephaly and cleft

palate (Dexter et al.,1980). Tumbleson et al. (1981a) found FAS minipiglets to weigh less than control piglets, but no differences in gestation length or total pigs born. Kubotsu et al. (2003) found decreased fetal survival and fetal weight in crossbred pigs. They observed the amount of alcohol that reached the fetus, and found positive correlations between blood ethanol concentrations with amniotic fluid and chorio-allantoid fluid, suggesting ethanol does cross the placenta and directly effects the development of the fetus. Most alcohol pig models seemed to only be used in research on the effects on the sow performance and nutrition, but Tumbleson et al. (1981a) was the first group to look at the ethanol consumption of the offspring as well. Piglets prenatally exposed to ethanol consumed more ethanol per kg per day for the first fifteen weeks of the test than control offspring. The offspring had been randomly housed together and given the same diet 12 weeks prior to ethanol consumption testing, suggesting the difference was from their fetal environment. No FASD research using the pig model has looked at behavioral deficits in the offspring prenatally exposed to ethanol. Therefore the objectives of the present studies were to first, determine the ethanol pharmacokinetics of gestating sows, and second, to assess coping strategy, anxiety, locomotion, and learning and cognition in domestic pigs prenatally exposed to ethanol throughout the entire length of gestation.

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CHAPTER II

SOW ETHANOL ELIMINATION STUDY

Introduction

Several factors can affect ethanol pharmacokinetics, including age, sex, body composition and whether or not a meal was consumed during consumption. Baraona et al. (2001) found women had higher and more prolonged alcohol levels than men given the same dose of ethanol. This may be attributed to slower gastric emptying in women and differences in body fat content. Robinson et al. (2002) also found females eliminated ethanol faster than males in both the brain and blood. Hormones affected alcohol dehydrogenase activity and ethanol metabolism (Thommasson, 1995; Lammers et al., 1995), with high progesterone and estrogen levels enhancing ethanol metabolic rates. Tumbleson et al. (1981) found that the estrus cycle influenced ethanol consumption levels in Sinclair (S-1) miniature sows as well. Therefore, differences in ethanol pharmacokinetics associated with pregnancy should be expected. Kubotsu et al. (2003) found cross bred pigs had higher blood ethanol concentrations (BEC) when they were pregnant compared to non-pregnant gilts. In the Sprague-Dawley rat, ethanol metabolism was actually greater among pregnant rats and lower blood ethanol concentrations (BEC) were observed (Badger et al., 2005). Badger et al. (2005) speculated that pregnancy may have protective state against ethanol toxicity by increasing metabolism.

Rats and mice have been used in alcohol research for many years, and many differences within the species and between the two rodent species has been reported. The pharmacokinetics of ethanol differ between the mouse and rat species; mice have a very sharp BEC rise with a rapid decline, while rats have a more prolonged rise and decline (Livy et al., 2003). Livy and coworkers (2003) suggests this difference between species should be considered when designing experiments so that the appropriate model is selected for the experimental questions being asked.

The pig is a novel model for alcohol and fetal alcohol research, and ethanol pharmacokinetics should first be determined in pregnant and non-pregnant pigs. A few investigators have found that the miniature swine model will freely drink ethanol to intoxication up to 16.3 g/kg (Dexter et al., 1976) even when water was available (Brown and Hutcheson, 1973). In young pigs, Chandler et al. (1989) and Keiding et al. (1979) found ethanol pharmacokinetics to be similar to humans, but in the perfused pig liver the ethanol elimination rate was about one to two- thirds of the human value (Damgaard et al., 1972; Keiding et al., 1979). These studies infused ethanol intravenously over a period of time. Although there are a few studies using the pig as a model for alcoholism, little is known about this model and the exact ethanol pharmacokinetics of pregnant sows. In order to justify the pig as a useful model for Fetal Alcohol Spectrum Disorder, the objective was to determine the ethanol elimination rate in gestating sows given a set dose of ethanol orally in order to reproduce normal absorption, distribution and elimination.

Methods

The pharmacokinetic elimination of ethanol in sows was determined. Eight sows were given 3 g/kg of 30 % ethanol orally through gavage. Sows were restrained by snaring the snout and a large animal fluid feeder with a plastic probe was used to administer ethanol directly into the stomach of the sow. Sows were food deprived the day of testing so that ethanol absorption would not be skewed by the contents of the stomach. Blood samples were taken every thirty minutes for two hours then every forty-five minutes up to eight and a half hours after gavage. BECs were determined using Gas Chromatography (GC) as described previously (Agrawal et al., 2013). Briefly, 20 µL of blood was taken via ear vein prick then put into 180 µL of nanopure water in a crimp-top glass GC vial to prevent ethanol evaporation. The samples were kept on ice during collection, and then transferred to a freezer. At time of reading, the samples were centrifuged for five minutes at 3 x g. Samples were analyzed using the headspace method (Finn et al., 2007). Known ethanol concentrations (0.0938, 0.1875, 0.375, 0.75, 1.5, and 3 mg/mL) were used to quantify BEC concentrations in an Agilent 7683 automatic liquid sampler GC (Agilent Technologies, Palo Alto, CA). Data were analyzed using the General Linear Models of SAS version 9.3 (SAS Inst. Inc., Cary, North Carolina).

Results

Figure 1 shows the BECs of each sow in the ethanol elimination study. We found large variation among sows. Sows differed significantly from one another ($P = 0.035$) and across time points ($P < 0.0001$). Sow 98 and 79 had greater ($P < 0.05$) BECs compared with the other sows. The initial phase of absorption was from 30 to

100 min, with most sows peaking between those times, except sow 73 who peaked at 4 g/dl at 180 min. Only a couple of sows showed an elimination phase during the time of blood collection. Figure 2 shows the BEC profile of all sows. Due to large individual variation in ethanol elimination among animals over time these data did not show a definite elimination phase for pregnant sows.

Discussion

High variation in ethanol absorption and elimination between sows in the present study were observed. Body weight differences between sows could be one factor that resulted in differences in ethanol metabolism; however the ethanol dose was based on body weight and therefore accounted for this. Body composition is known to affect ethanol pharmacokinetics, and if there were differences in body composition between sows then ethanol metabolism rate differences could be seen (Robinson et al., 2002). The percentage or distribution of fat between sows or give them composition scores were not measured in the present study, so the differences in body fat composition were not known.

The high variation among sows in regards to BEC levels could reflect the location of blood collection, or differences in the amount of ethanol that the sow received. Sows received 3 g/kg of 30% ethanol but the nature of administering the ethanol was difficult and some liquid was lost. The rate of ethanol absorption into the blood was different for each pig and the variability between pigs makes it hard to discern how long it took to reach peak BECs. The long plateau phase may reflect delayed absorption possibly from food left in the stomach; however pigs had not been

fed the day of the study. Not only was absorption variable but so was ethanol elimination. Only a few sows showed an elimination phase by 550 min (Figure 2), and perhaps elimination had just begun. A longer sample time may have shown the elimination phase for more animals. The mouse takes 360 min and the rat 450 min to reach zero at 3.8 g/kg of 21% ethanol (Livy et al., 2003), but both of those models have a very high metabolism rate compared to humans, so a longer testing time may be needed in the pig which is more similar to the human (Chandler et al.; 1989; Keiding et al., 1979). Kubotsu et al. (2003) found BECs reached a peak of 230 mg/dl after consuming ethanol in the diet of peripubertal crossbred pigs. Ten hours later, before the next feeding, BECs had not reached baseline, suggesting we should have increased our blood collection time. However, the methodology used by Kubotsu et al. (2003) differed from the present study as a ration of ethanol was given in a meal and it is known that consuming ethanol with a meal decreases the absorption rate.

During the entire blood collection phase, sows showed signs of drunkenness and did not appear to have completely sobered up at the completion of blood collection. During the entire sampling time, sows laid down, but if they did sit up they would sway and have difficulties making postural changes. Two sows got out of their pens and when moving them back in, they were stumbling and leaning against objects while walking.

For the most part, sows were drunk enough that blood collection did not disturb them and it was easy to take blood from the ears while they remained lying. This passivity due to intoxication was also seen by Dexter et al. (1976). In the present

study, two sows vomited 20 to 30 min after ethanol administration. Those two sows showed signs of aggressiveness during the elimination phase, and even when they were still laying down sleeping, they were easily irritated by the investigators trying to get blood for BECs. One sow charged the investigators and escaped her pen. Dexter et al. (1976) observed similar aggressive behaviors during withdrawal periods in pigs. Blood collection on both of those sows ended early because handling them and collecting blood became too difficult. One of the sows that vomited was # 93 and the elimination phase had begun although blood collection on her ended early. She started the elimination phase sooner than other sows because of losing a large quantity of ethanol from vomiting.

In future studies it would be advantageous to do a pilot study to determine the best place for blood collection to measure BECs in pigs. Rodent models often use the tail or eye orbit for blood collection, and location is known to affect BECs. The jugular vein is the most common place for pig blood collection; however this location was not chosen in the present study because the pigs were lying down, which would have made collection difficult. In the future, both a jugular and ear vein catheters could be inserted, then which location is better for measuring BECs could be tested. Similar to the present study, Dexter et al. (1976) found intoxicated pig ears to become flaccid which could make blood collection in this location difficult. Reducing the dose may also help reduce the time necessary to complete a full elimination.

The maximal ethanol elimination rate in young pigs and in the perfused pig liver was similar to the human; 0.62 to 1.63 and 0.80 to 1.26 mmol/min/kg

respectively (Keiding et al., 1979). So the pig is a good model for ethanol pharmacokinetic studies. Differences between intraperitoneal (i.p.) and intragastric (i.g.) gavage have been found, with i.p. resulting in higher BECs in mice and rats (Livy et al., 2003). Previous ethanol elimination studies in pigs (Chandler et al.; 1989; Keiding et al., 1979) have used intravenous infusion of ethanol over longer periods of time rather than a bolus of ethanol. However, oral gavage is probably the most feasible method of ethanol administration in an animal the size (228 kg) of the sows in this study. At a dose of 3 g/kg of 30%, an average of 2.7 L of ethanol was administered to the sows. Some liquid was lost during gavage, but not with every pig, and were minimal amounts. The difference in exact administration of ethanol could also be an indication of the variation of these results. The sows received large amounts of ethanol and only small amounts of liquid were lost in comparison, but even small amounts reduced the dose from 3 g/kg.

Restraint can induce stress in an animal which can affect ethanol absorption. Stress decreased the amount of ethanol absorbed and eliminated (Benet, 1992) by inhibiting the digestive system (Guyton, 1991). Even acute stress reduced perceived intoxication and altered the BEC curve in humans (Breslin et al., 1994). Ethanol administration techniques in the present study did get quicker after the first couple of sows, but the large volumes of liquid administered required the animals to be restrained much longer than under normal conditions, and could have affected the rate of absorption resulting in the variation of these data. Future studies could habituate the animals so that restraining and gavaging were not stressful procedures.

In conclusion, 3 g/kg of 30% ethanol resulted in high BEC levels equivalent to illegal intoxication in humans. However, there was high variation in the absorption and elimination rates between pregnant sows, and BECs showed a long plateau phase with no clear elimination phase. The BEC data were up and down in nature and more animals are needed to determine ethanol pharmacokinetics in pregnant sows.

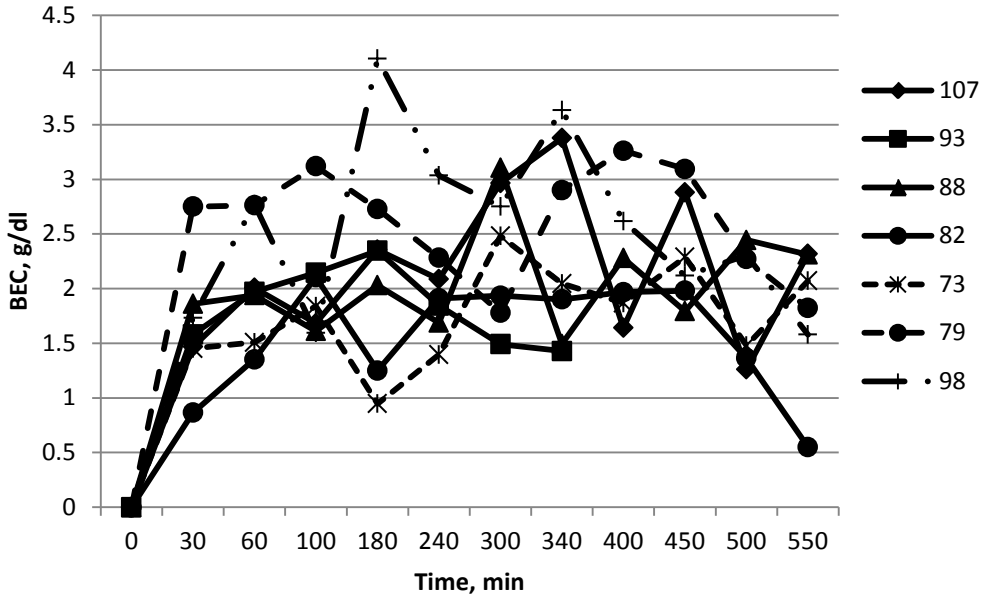

Figure 1. Blood Ethanol Concentration (BEC) of individual sows given 3 g/kg of 30% ethanol by intragastric gavage.

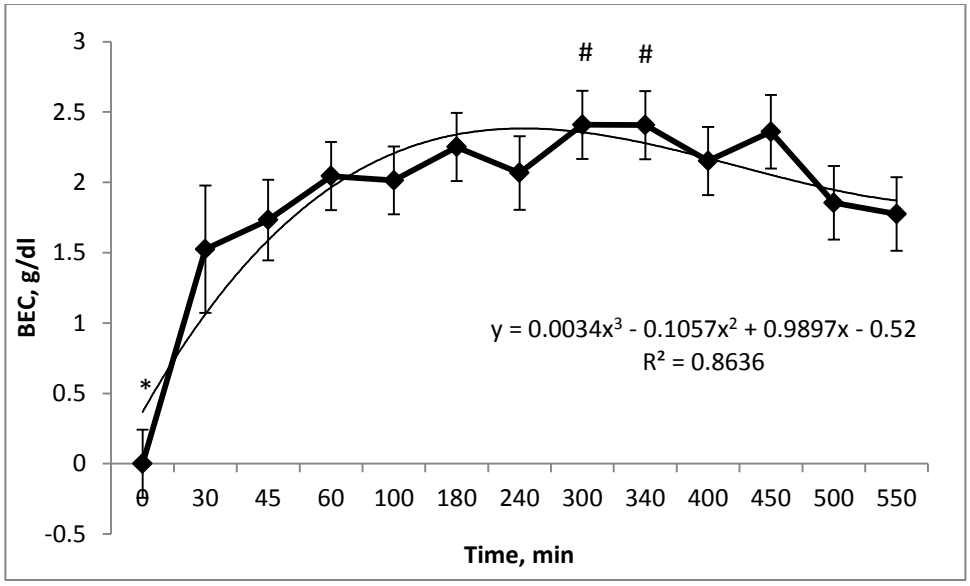

Figure 2. Blood ethanol concentration (BEC) profile for sows that received 3 g/kg of 30% ethanol by intragastric gavage. Data are presented as LSMeans ± SEM. An asterisk, * represents time 0 was significantly ($P < 0.05$) different from all time points. Times 300 and 340 tended (#; $P < 0.10$) to be different from 30, 45, 550 min.

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CHAPTER III

BEHAVIORAL IMPLICATIONS OF OFFSPRING IN A FREE CHOICE ETHANOL SWINE MODEL

Introduction

Drinking alcohol during pregnancy can have detrimental outcomes on the offspring. Ethanol is the most common, most preventable teratogen, a factor that causes birth defects and abnormalities in the developing fetus (Chung, 2005; Thackray and Tifft, 2001). This prenatal exposure to ethanol can lead to a spectrum of developmental, physical, psychological and behavioral disorders that fall under Fetal Alcohol Spectrum Disorder (FASD).

The degree of anomalies that present with FASD depend on many factors including the amount of ethanol that crosses the placenta to the fetus (Riley and McGee, 2005), and peak BECs (Thackray and Tifft, 2001; Bonthius and West, 1990). The dose and timing of exposure compared to fetal developmental milestones affects which structures will be altered and to what severity (Foltran et al., 2011; May and Gossage, 2011; Riley and McGee, 2005). The most severe effects occur in early gestation during the gastrulation stages of embryogenesis and organogenesis when cells are rapidly differentiating. However, neuronal loss has been found in the second (Miller and Potempa, 1990) and third (Bonthius and West, 1990) trimesters as well. The third trimester is susceptible to ethanol exposure because the brain has its final growth spurt when alcohol can damage the cerebellum, hippocampus, and the prefrontal cortex (Coles et al., 1991).

The drinking paradigm for individual mothers varies greatly and leads to numerous degrees of severity of prenatal alcohol exposure (PAE). The spectrum can range from children not being affected or only to a low degree, Fetal Alcohol Effect (FAE), all the way to Fetal Alcohol Syndrome (FAS) at the severe end. There are three distinct anomalies that must be present for a FAS diagnosis: pre and/or postnatal growth deficiency, facial anomalies, and CNS dysfunction (Jones and Smith, 1973). The facial anomalies are microcephaly, a flat midface, and a poorly developed philtrum and thin upper lip. Central Nervous System dysfunction includes neurological abnormalities, developmental delay, hyperactivity or intellectual and cognitive impairment (Jones and Smith, 1973; Thackray and Tifft, 2001), and may be some of the most severe birth defects because the brain is highly susceptible to the teratogenic effects of ethanol (Chang et al., 2003). All three anomalies must be present to meet the criteria for diagnosis of FAS and many children do not meet it because they lack facial dysmorphology, and this is the reason why many individuals fall under the broad spectrum of FASD.

Offspring with FAS have a small brain (Jones and Smith, 1973). This may be due to decreases in specific regions of the brain that are most susceptible to ethanol, such as the basal ganglia, corpus callosum, and hippocampus (Mattson et al., 2001). Specific brain regions affected by PAE not only have a decrease in size and volume, but can change location, have a decrease in fiber tract number and connectivity that may not be uniform across the structure (Mann et al., 2001; Nuñez et al., 2011; Riley and McGee, 2005; Wacha and Obrzut, 2007; Davis et al., 2011).

Even if facial dysmorphology is not present, brain dysmorphology can be leading to many neurobehavioral disturbances. Prenatal exposure to ethanol is one of the most common non-genetic causes of mental retardation. The structures of the brain most susceptible to ethanol include areas responsible for attention, spatial and verbal memory, executive functioning (cognitive flexibility, response inhibition, planning, reasoning, working memory), motor control, learning, memory formation, and emotional and social dysfunction (Nunez et al., 2011; Mattson et al., 2001; Roebuck et al., 1998; Davis et al., 2011; Kodituwakku, 2007). These behavioral phenotypes help with identifing the various levels of diagnoses under the umbrella term FASD, however no single behavioral phenotype has been identified making diagnosis difficult and questionable at the clinical level (Astley et al., 2009).

Children with FASD display social impairments such as poor judgement, inability to understand consequences, and impulsivity (Mattson et al., 1999; Davis et al., 2011). Adaptive skills are social skills necessary for individuals to establish and maintain relationships, and areas of social competence (Mattson and Riley, 2000) and social relationships may be altered in FASD (Thomas et al., 1998; Kully-Martens et al., 2012). Mattson and Riley (2000) found children with FAS and PAE had more social, attention, and aggressive behavioral problems than childen not prenatally exposed to ethanol from the Child Behavior Checklist questionnaire. Specific behaviors scored in each of these areas are clinging and not getting along with others, concentration and impulsiveness, and fighting and attacking others, respectively. The Vineland Adaptive Behavior Scale questionnaire has been used to analyze different

domains of social skills. Individuals with FASD are impaired in interpersonal relationships, coping skills and the use of play and leisure time, which may escalate with age (Thomas et al., 1998). Social problems can lead to peer rejection, which in turn can contribute to increased aggression, anxiety and depression, antisocial behavior, and addictive behaviors that are also typically observed behaviors in FASD (Kully-Martens et al., 2012).

Individuals with FASD are inattentive and hyperactive, and are often compared to children diagnosed with ADHD. However, it is thought that the dimensions of attention are different in ADHD and FASD individuals. Individuals with ADHD have difficulty sustaining and focusing on tasks, while FASD difficulty is seen in the ability to learn and manipulate new material in working memory while being processed into long term memory (Coles, 2001; Coles et al., 1997). The distractibility scales in a temperament test found FASD children to not be distractible; they cannot disengage from ongoing tasks (Nulman et al., 2004). Hyperactivity in rodent models have shown that Wistar (Ledig et al., 1990) and Sprague-Dawley (Hellemans et al., 2010) rats cross more squares and are more active in an open field test. Increased locomotor activity can be associated with increased anxiety and fear (Carola et al., 2002), but the tests used to measure locomotion also assess anxiety in animal models. Anxiety can have effects on locomotor and exploratory behaviors, and may also be a reason some studies observed both reduced locomotion (Hellemans et al., 2008; Kleiber et al., 2011) and no differences in locomotion (Brocardo et al., 2012). Carola and coworkers (2002) compared different mouse strains in an open field test and elevated plus maze,

and found that C57BL/6 mice show anxiety-dependent locomotor and exploratory activity, while BALB/c mice showed depressed locomotor activity and were more anxious explorers.

Prenatal exposure to ethanol causes poor coping adaptations, hyperresponiveness of the HPA axis to stressors, and deficits in HPA recovery (Weinberg, 1988; Bilitzke and Church, 1992; Weinberg et al., 1996). Hyperactivity and dysregulation of the HPA axis causes greater rates of anxiety disorders and depression in FASD (Hellemans et al., 2008). Conflicting studies have found rats to be both less immobile (Bilitzke and Church, 1992) and more immobile (Hellemans et al., 2010; Brocardo et al., 2012; Carneiro et al., 2005) in forced swim tests. However, both results are indicative of anxiety: either being unable to calm down once excited, or giving up when escape is impossible, respectively. There are different types of anxiety and strategies for coping with that anxiety. Active coping is important for psychological well-being, while avoidant coping is related to higher maladjustment (Thorne et al., 2013). The elevated plus maze is a measure of anxiety with rodents spending less time in open arms opposed to closed arms (Brocardo et al., 2012; Dursun et al., 2006). Osborn and coworkers (1998) divided the elevated plus maze into exploration and fear factors. The fear factor indicated PAE females show high levels of fear and spent less time in open arms, made fewer open arm entries than controls, and had higher levels of corticosterone in response to the elevated plus maze but not basal levels.

It seems contradictory that FASD leads to increased anxiety and poor social skills, but increases exploration and novelty seeking, as anxiety disorders and social skill deficits would generally make one think of introverted personalities and avoidance of anxious situations. But poor judgment, inability to understand consequences, impulsivity and novelty seeking are found in FASD individuals at all levels of development and into adulthood (Mattson et al., 1999; Davis et al., 2011; Thackray and Tifft, 2001). Children with FASD seek out strangers and novel surroundings, are more adaptable to new situations and less fearful of change (Nulman et al., 2004). Animal models have shown less avoidance to novelty by spending more time in novel compartments compared to familiar compartments (Ledig et al., 1990) and spending more time in the center of an open field when a novel object was present, as well as displaying more exploratory activity (Allan et al., 2003).

High impulsivity, poor judgement, aggression and antisocial characteristics increase the development, and predictability of substance abuse disorders in adolescents with FASD (Fryer et al., 2007; Gunzerath et al., 2011). Postnatal responsiveness to ethanol is increased by fetal ethanol exposure through programming of the reward circuitry in the brain (Foltran et al., 2011) and associative learning during the first drinking experience (Pautassi et al., 2012).

The hippocampus and basal ganglia are involved in learning and memory and are particularly sensitive to the teratogenic effects of ethanol; therefore several areas of learning and memory are affected by PAE. Intellect and neuropsychological measures are used to provide insight into cognitive deficits in human studies. Tests are

chosen based on sensitivity of the specific brain region function deficits under investigation.

Individuals with FASD have difficulties encoding verbal information and with response inhibition capabilities (Mattson et al., 1996b). They learn fewer words and have difficulty in verbal memory recall (Mattson et al., 2011; Kerns et al., 1997). Observed deficits in learning are thought to be in acquisition of the material and not in the ability to remember the material over time (Mattson et al., 2001), but different types of recall may be affected. Individuals with FASD have greater difficulty with free recall and recognition recall (Crocker et al., 2011; Mattson et al., 1996b; Kodituwakku, 2007; Kerns et al., 1997). A delay period after learning may lessen recall memory (Mattson et al., 2011).

A lower rate of learning and recall after a delay period are also observed in FASD affected nonverbal learning and memory (Mattson et al., 2011). Visual-spatial memory falls under the nonverbal learning domain. In general, more trials are required to learn (Riley et al., 1979) and more errors in reference and working memory are made (Zimmerberg et al., 1991). Performance depends on the type of navigation used. More errors are observed in spatial conditions compared to cued conditions (Berman and Hannigan, 2000; Hamilton et al., 2003; Kelly et al., 1988).

Not only is the brain and behavior affected through PAE, but the immune system regulation is as well. Alcoholism causes an individual to be immunodeficient and more susceptible to infections (Cook, 1998; Roselle et al., 1993). When a pregnant woman drinks throughout pregnancy her offspring's immunity becomes

compromised, both from her suppressed immunity and from the direct and indirect effects of ethanol crossing the placenta. Prenatal exposure to ethanol causes viral and fungal infections from cellular immunity deficits, while humor immunity deficits can increase sepsis, meningitis and pneumonia (Zhang et al., 2005; Johnson et al., 1981; Grossmann et al., 1993).

Deficits in adaptive immunity include low T-cell counts, decreased lymphocyte proliferation and decreased immunoglobulins (Gottesfeld and Abel, 1991; Zhang et al., 2005). Reduced thymus weight and size and alterations in the cortex and medulla ratio or lack of delineation between the two (Budec et al., 1992; Ewald and Walden, 1988) could be responsible for deficits in lymphocyte proliferation and production. An immature thymus may be unable to elicit an efficient immune response to a pathogen. In both C57BL/6 mice fetuses (Ewald and Frost, 1987) and 90 day old offspring of Sprague-Dawley rats (Weinberg and Jerrells, 1991) prenatally exposed to ethanol, the number of thymocytes and lymphocyte response to a mitogen were decreased.

The innate immune response is responsible for eliciting the immediate activation of phagocytic cells to locate and kill pathogens so that they can present antigens to the adaptive immune system. Deficits in innate immunity from fetal alcohol exposure are not always found (Zhang et al., 2005; Grossmann et al., 1993; Johnson et al., 1981), although a few studies have shown low counts of eosinophils and neutrophils (Gottesfeld and Abel, 1991) and decreased phagocytosis in macrophages (Gauthier et al., 2005; Ping et al., 2007). More research in to how PAE

impairs the immune response is needed. The rodent model may not be a good immune model for the human. However, the pig immune system is 80% similar to humans and may make a good model for the affects of alcohol on immunity.

The domestic pig is very similar to humans in anatomy, physiology, immunology, and behavior and could be an advantageous model in translational research. The domestic pig is more similar to humans than the rodent model and less expensive than nonhuman primates, and does not present with as strong of ethical concerns because it is still widely seen as a production animal (Gieling et al., 2011).

Ethanol elimination is similar in the pig and human liver (Keiding et al., 1979), enabling the pig to be a good model for pharmacokinetic evaluation of ethanol (Gieling et al., 2011). One group of investigators established the Sinclair miniature pig as a good model for FASD (Dexter et al., 1980; Dexter et al., 1976, Tumbleson et al., 1981a, Tumbleson et al., 1981b). These sows voluntarily drank ethanol up to 30% w/v and went through states of both intoxication and withdrawal. The offspring of second generation drinking sows showed facial anomalies characteristic of FAS such as microcephaly and cleft palate and lower birth weights. However, to our knowledge, FASD pig offspring behavior has not been evaluated.

Therefore, the objective of the present study is to develop a free-choice domestic pig model for FASD, focusing on the behavioral consequences of fetal ethanol exposure on the offspring. We hypothesize that the nursery age FASD offspring will show more signs of activity and novelty seeking in an open field test, be less social, and therefore, be submissive in a social interaction test, and have decreased

reference and working memory as shown by an increase in errors and latency to reach a reward using an adaption from the Hebb-Williams maze. At the equivalence of adolescence, we hypothesize that FASD pigs will drink more than control pigs in both individual and social settings.

Methods

Sows

Fetal Alcohol Model

Four replicates were used for a total of eight ethanol consuming sows and eight control sows (2 sows/trt x 2 trt = 4 sows x 4 replicates = 16 sows total). Sows were in their second parity (PIC USA Camborough-22 sow line; 182 ± 5.60 kg average body weight). Before breeding, 10 sows per replicate were given access to ethanol. The sows that drank more than 1.5 L of 20% ethanol were then used in the study. Ethanol sows (ETOH: n = 8) were given *ad libitum* 20% ethanol daily from day one of gestation (day of breeding) until farrowing (114 days). Control sows (CON: n = 8) did not have access to ethanol at any point during gestation. The amount of alcohol consumption was checked daily as well as signs of drunkenness (vomiting, swaying, flushed face and ear, hiccups, passivity, decrease in muscle tone), and overall health and welfare (body condition, feed consumption). Sows were restrained by snaring and blood samples were drawn via jugular puncture (5 ml in EDTA) at 0, 30, 60 and 90 days of gestation to measure the stress response (cortisol), total leukocyte counts and differential (ProCyte Dx Hematology Analyzer, Idexx Laboratories Inc., Westbrook,

Maine), and neutrophil oxidative burst (OB) (Cell Lab Quanta SC flow cytometer, Beckman Coulter, Fullerton, CA), and for blood ethanol concentrations (BECs) to drinking during pregnancy.

Reproductive parameters

Sows were moved into farrowing crates five days before farrowing. Pre- and post-farrowing body weights were taken. At farrowing, reproductive parameters were taken (total number born, born alive, stillborns, mummies, total born dead, percent of boars and gilts, and number weaned), and the offspring were checked for anatomical deformities. Stillborn pigs were fetuses that were born dead and usually die later during development, while mummies were fetuses with a dark, petrified appearance that typically died early during development but were too large to be reabsorbed by the dam, and total pigs born dead were either stillborns or mummies.

Offspring

Weights were taken at birth, weaning (21 d) and finishing before marketed (180 d). The female offspring were used in behavioral test for dominance, locomotion, anxiety, learning and cognition, and drinking behavior. Only female offspring were used to reduce variation because it is known that sex differences exist in the behavioral response of PAE rodent models.

Experiment 1

Offspring remained with the birth dam during the entire lactation period. Offspring from ethanol sows were noted as ethanol (ETOH) piglets and offspring from control sows were control (CON) piglets. This allowed sows to raise their own offspring and not induce stress caused by cross fostering into the model.

Processing

At three days of age, all pigs in the litter (CON: n = 97; ETOH: n = 93) were processed as is routine in the pig industry. Processing involved ear notching (for litter and piglet identification), teeth clipping, tail docking, an iron shot (100 mg), and castration for males. Processing was recorded using camcorders (DCR-SR85 60GB Handycam Camcorder, Sony, New York) and stress vocalizations were analyzed using an automatic stress call monitoring system (STREMODO, Forschungsinstitut für die Biologie landwirtschaftlicher Nutztiere, Dummerstorf, Germany). The percentage of stress vocalizations in response to handling and during each individual procedure were analyzed.

Temperament

At five days of age, all of the offspring (CON: n = 95; ETOH: n = 90) were given a back test to measure coping ability (Hessing et al., 1993). The piglet was placed on its back in a supine position in a v-shaped trough for one minute. The handler loosely held the neck back with one hand and loosely held the hind legs with the other. The number of escape attempts (continuous series of wiggles), duration of

escape attempts and vocalizations were recorded. Escapes were classified as being more than two seconds apart or the pig being secured back down by the handler before lifting again. Vocalizations were considered one if less than two seconds separated them. Each piglet was classified as a High Responder (HR) if it tried to escape three or more times, an Intermediate Responder if it escaped or vocalized two times, or a Low Responder (LR) if it made an escape attempt or vocalized one time or less.

Baseline blood measures and teat order

At seven days of age, the entire litter was removed from the dam for baseline stress (cortisol) and immune (oxidative burst (OB) and leukocyte counts and differential) measures. Pigs were placed in the supine position in a v-trough. A handler held the front and hind limbs to secure the pig, while the bleeder held the head back with one hand. Blood was taken via jugular puncture (2 mL EDTA and 2 mL Heparin) in vacutainers.

Immediately after bleeding a number was written on each pig's back for identification purposes to establish teat order for litter dominance. Only the offspring also used in the socialization test and food competition test were included in the analyses (CON: n = 29; ETOH: n = 29). Once the piglets were returned to the sow the nursing order was recorded. Paired teats were numbered cranially (one) to caudally (usually seven depending on the number of working teats) (Fraser and Jones, 1975). The piglets on the most cranial teats were considered dominant, and classified as 1, to

the piglets at the caudal teats, classified as 3, while all positions in between were classified as 2.

Isolation

At fourteen days of age female offspring (CON: n = 26; ETOH: n = 26) were placed in a 1.2 x 1.2 meter pen by themselves for two hours to assess the stress response to isolation. Video (Sony) was recorded and analyzed on Observer 10.5 (Noldus) for maintenance behaviors (walk, stand, sit), escapes and time to "relaxation". Blood via jugular puncture (3 mL in Heparin) was taken before and after the tests period to assess cortisol concentrations.

Social Interaction

At least two days (16 days of age) after completion of the isolation test, pigs (CON: n = 29; ETOH: n = 29) were socialized with an unfamiliar pig of the opposite treatment (CON-ETOH). Two pigs were placed in a 1.2 x 1.2 meter pen in a room with no visual contact with other pigs or people, for two hours and video recorded (Sony). The pig that was more aggressive (biting, chewing and pushing the head or body) and that won the most fight bouts, a series of social encounters that ended when one pig no longer retaliated and then the interaction stopped, was considered dominant, while the pig that won the least number of fights and displayed escape behaviors, submissive.

Weaning

At weaning (21 d), all offspring were weighed and female offspring were housed in nursery pens (1.2 x 1.2 m) with one piglet from each litter (CON and ETOH piglets were mixed). Blood was taken at 0, 4 and 24 h after weaning to measure the stress response (cortisol) and the immune response at 0 and 24 h (white blood cell (WBC) and differential) to weaning. These gilts were used in all behavioral tests starting one week after weaning.

Locomotion and anxiety

One week after weaning (four weeks of age) female pigs (CON: n = 17; ETOH: n = 17) were subjected to the open field- approach test to measure overall activity, and anxiety and fearfulness to a novel object and environment. As adapted from Thodberg et al. (1999), pigs were placed in a 2.4 x 2.4 meter arena that was divided into nine equal squares with paint on the floor. The pig was placed in one corner of the arena to start the test. There was a five minute exploration period for the pig to explore the arena. Then a novel object (bucket) was descended from the ceiling for another five minute interaction period. Video equipment (8 Channel H.264 Networkable Security DVR, Supercircuits, Austin, TX) recorded the test for the number of squares entered in both periods (exploration and interaction), duration in each square, duration in the middle of the arena, time to approach the novel object, duration of interaction, number of interactions and type of interaction (play or aggressive). Blood samples via jugular puncture (3 mL in EDTA) were taken directly

before and after the open field- approach test to measure the stress response (cortisol) to a novel environment and object.

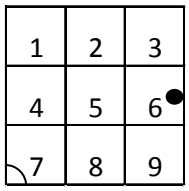

Figure 3. Layout of the open field-approach test. The round line represents the entrance, and filled in circle where the bucket is placed. The numbers represent the individual squares.

Learning and cognition

To measure learning capabilities, five week old female pigs (CON: n = 33; ETOH: n = 25) were trained to find a reward in a maze. Initially, pigs were acclimated to the test arena by randomly placing the pigs in an open arena measuring 5 x 3 meters without internal barriers. The maze arena was surrounded by black plastic so pigs could not be influenced visually by a human or another pig. The acclimation period was designed to allow the pigs to become familiar with the arena prior to the introduction of internal boundaries, and so that the maze is not a novel environment. A cookie reward was placed in the top right corner of the arena, diagonal from the start position. (A sweet reward was used to keep pigs from being food deprived prior to maze testing). The food placement stayed constant during all subsequent maze tests. Pigs were subjected to the acclimation period two times a day for two days at two hour intervals for a total of four trials. Pigs were placed in the bottom left corner

of the arena and given fifteen minutes to find the reward in the first two trials and ten minutes in the last two trials. If the reward was not located by the pig in the allotted time, they were led to the reward and the maximum time score was given. Urine and feces was picked up after each pig.

At the completion of the acclimation period, pigs were tested in Maze I for learning and for cognition (Maze I Memory). The reward remained in the same place as during acclimation, but internal barriers were placed inside the arena (de Jong et al., 2000). The pig had ten minutes to navigate its way to the reward. If it did not find it in the allotted time it was led to the reward, and the maximum time score of ten minutes was given. Pigs were subjected to Maze I, two times a day for two days at two hour intervals for a total of four trials.

Five days after completing Maze I, the pigs were tested again in Maze I for cognition, and referred to as Maze I Memory. Pigs were tested twice in one day at two hour intervals, for a total of two trials. Ten minutes was allotted to find the reward. The pig was led to the reward if they had not approached it in the allotted time, and the maximum time score of ten minutes was given.

After the completion of Maze I Memory testing, pigs were tested in Maze II. Maze II was used to see if the pigs could still learn while their long term spatial memory was being assessed. Similar to Maze I, the reward remained in the same place as in the acclimation period, but the internal barriers were changed again. Pigs were subjected to the Maze II two times a day for two days at two hour intervals, for a

total of four trials. If the pig did not find the reward in ten minutes it was led to the reward, and the maximum time score of ten minutes was given.

Each maze session was video recorded (Supercircuits). The latency to the reward and frequency of defecation and urination were measured for all maze types. When internal barriers were used, the latency to the reward, locomotion, and the duration and number of errors (when an imaginary line was crossed going in the wrong direction of the reward, i.e. cul-de-sac or blind alley) were recorded. It was not counted as an error when the pig walked out of the error zones.

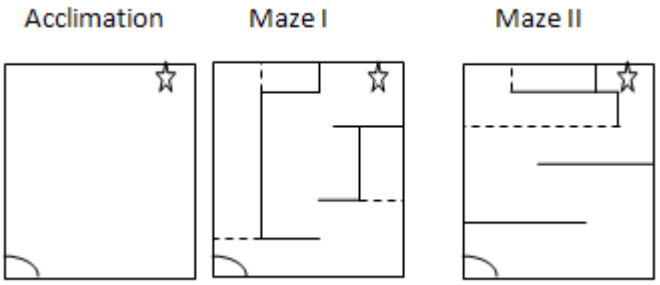

Figure 4. Layout of each maze type, acclimation, maze I and maze memory, and maze II. Curved lines represent the entrance, and the star the placement of the reward. Solid lines are internal barriers, and dotted lines are imaginary lines that represent "wrong-turns."

Food competition – finishing pig dominance

At eight weeks of age, pigs (CON: n = 29; ETOH: n = 29) were moved into the finishing barn in pens (3.7 x 1.1 m) with slatted concrete flooring. Feed and water was provided *ad libitum* except during testing as described below. Pigs were group housed in groups of four in the finishing pens with two pigs per treatment, so that groups from weaning were kept intact.

A food competition test was performed to determine the pen dominance order at finishing size, approximately 150 days of age. Pigs were fed in meals for two hours a day for two days so that they would be hungry at the time of the test. A pair of pigs had access to feed for two minutes. The total time of each pig eating from the one 0.15 m feeder slot was recorded. The pig that spent the most time eating, or performed the most displacements (physically removing another pig from the feeder) was considered dominant over the other pig. Every possible pair of pigs in the pen was tested against one another.

Experiment 2

Some female offspring were cross fostered within twenty-four hours to a dam of the opposite gestation treatment throughout the lactation period. Cross fostering allowed the variable of an "alcohol-abusing" rearing maternal effect to be removed (Kelly et al., 2000). Offspring from six of the sixteen sows (CON: n = 3; ETOH; n = 3) were used in experiment 2. Each sow only nursed two treatments, piglets she birthed and those from the opposite treatment. Test piglets were noted by both the gestation and lactation treatment, respectively. Control-Control (CC: n = 5) piglets were from CON sows and remained with their birth mother throughout lactation. Control-Ethanol (CE: n = 5) piglets were from CON sows but were moved to an ETOH sow twenty-four hours after birth throughout the lactation period. Ethanol-Control (EC: n = 5) piglets were birthed from ETOH sows, then cross fostered to a CON sow during lactation, and Ethanol-Ethanol (EE: n = 5) piglets were from ETOH

sows but remained with the birth sow throughout lactation. All experiment 2 pigs went through the same procedures and behavioral tests as experiment 1 pigs mentioned above. However, only these groups of pigs were assessed for drinking behavior at adolescence.

Drinking behavior

At six months of age (adolescent equivalencies to human development (Bazer et al., 1988)), gilts from experiment 2 were given 20% ethanol in water for forty-eight hours to determine the drinking patterns of offspring exposed to ethanol during development and whether or not they drink more than the control offspring in both individual and group pens. This group of offspring were labeled using a dual letter system by gestation litter (first letter) and lactation litter (second letter). Some gilts were cross-fostered to litters of the opposite treatment or remained with the birth sow (CC: n = 5; CE: n = 5; EC: n = 5; EE: n = 5). Pigs were color coded (Prima Tech Prima Glo Marking Spray, QC Supply, Schuyler, NE) for identification. Video cameras (Supercircuits) were zoomed in on the drinker to determine which pig was drinking. A flow meter (MeterMall USA, Marysville, Ohio) was used to determine the amount of ethanol volume drunk.

Blood analysis

Whole blood was analyzed to determine total leukocyte counts and percentages (Idexx Laboratories Inc.), and the neutrophil to lymphocyte (N:L) ratio was calculated

by dividing the percentage of neutrophils by the percentage of lymphocytes. A subset of offspring for basal (CON: n = 54; ETOH: n = 47) and weaning (CON: n = 16; ETOH: n = 16) differentials were analyzed.

The OB capacities of peripheral blood neutrophils in response to enteropathogenic *Escherichia coli* were analyzed according to Hulbert et al., 2011a, b. In short, whole blood was incubated in an ice bath for fifteen minutes and then dihydrorhodamine and fluorescently labeled *E. coli* were added to each sample, and incubated in a water bath (38.5^{o}C) for ten minutes. Samples were placed in an ice bath for five minutes to stop the reaction, and then red blood cells were lysed and washed. Leukocytes were analyzed by dual-color flow cytometry (Beckman Coulter). Analysis software (QuantaSC MPL, Beckman Coulter) gated neutrophils for OB and geometric mean fluorescence intensity (GMFI).

Whole blood was centrifuged for 15 min at 1,200 x g, plasma was collected and stored at -20^{o}C until analyzed for cortisol. All plasma samples were analyzed in duplicate. Circulating concentrations of cortisol were determined using a commercially available competitive-binding ELISA kit (Enzo Life Sciences Inc., Farmingdale, New York). The intra- and inter-assay coefficients of variation were 5.10 and 0.23%, respectively. Sow cortisol was only analyzed at days 0 and 60 of gestation when drinking levels were high, and a subset of offspring (CON: n = 17; ETOH: n = 21) cortisol was analyzed before and after the social interaction test.

Statistical analysis

All data in both experiments were tested for constant variance and departures from normal distribution. Normality of the residuals was confirmed by evaluating the Shapiro-Wilk statistic using the Univariate procedure of SAS version 9.3 (SAS Inst. Inc., Cary, North Carolina). Data lacking normality were transformed logarithmically using $\log_{10}$. Measures that required transformation included cortisol concentrations, various leukocyte counts, sow OB mean, percent stress vocalizations for handling, isolation behaviors, various open field test duration measures, various maze duration measures in both experiments, and offspring group drinking time and number in experiment 2. All repeated continuous data were analyzed by restricted maximum likelihood analysis of variance using the Mixed model procedure of SAS (SASv9.3). Categorical and binomial data (back test response category, dominance status, completion of mazes) were subjected to analyses using the General Linear Mixed Models and/or FREQ procedures of SAS (SASv9.3).

For the sow reproductive parameters, number of total pigs born per litter was tested as a covariate for mummies, stillborns, total born dead pigs per litter and birth weight. The covariate was removed from all analysis except for birth weight, and birth weight was used as a covariate for wean weight. Baseline cortisol for sows and social test were examined as a covariate. The covariate was removed from the sow data when found to be not significant, but used in the offspring social test. No sex effects were found in the back test and were taken out of the model. Least square means ± standard error of the means are presented in the results.

When data was collected over time (e.g. sow consumption, cortisol concentrations, immune measures, open field behaviors) the model used a repeated measures over time statement. The subject of the repeated statement was the sow nested within treatment for sow data, litter within treatment for experiment 1 and litter for experiment 2. The model was run with all available covariance structures for the within-subject measurement. The appropriate covariance structure was chosen for each analysis based on the Schwarz's Bayesian Information Criterion. Degrees of freedom for F-tests of the fixed effects were estimated using the Kenward-Rogers approximation.

Experiment 1

For all analyses in experiment 1, litter nested within treatment was a random effect. This study used a Completely Randomized Design with two treatments levels, CON and ETOH. The experimental unit was litter within treatment. Data from the two studies were combined in a larger dataset. In experiment 1, all pigs were considered.

Experiment 2

For all analyses in experiment 2, litter was a random effect and a complete block. Experiment 2 used a Completely Randomized Block Design with four treatment levels, CC, CE, EC, and EE.

Results

Sows

Drinking

Figure 5 illustrates the average ethanol consumption during sixteen weeks of gestation. From weeks 1 – 10 of gestation, the average 20% ethanol consumption was over 2 g/kg body weight. Sow consumption ranged from an average of 0.85 to 2.78 g/kg of 20% ethanol throughout gestation. The lowest daily intake of any individual sow was 0.17 g/kg and the highest 6.17 g/kg. The least amount of consumption was towards the end of gestation and dropped below an average of 2 g/kg at eleven weeks of gestation, however, after peak consumption at nine weeks of gestation ethanol consumption decreased.

HPA and Immune response

There were no differences ($P = 0.12$) between the cortisol concentrations of CON and ETOH sows at 0 or 60 d of gestation (CON: 19.0 ± 2.60 ng/mL; ETOH: 14.0 ± 2.60 ng/mL). However, there was a tendency ($P = 0.086$) for cortisol concentrations regardless of treatment to be higher at 60 d of gestation (0: 12.4 ± 2.76 ng/mL; 60: 20.6 ± 2.76 ng/mL). There was no interaction between treatment and period ($P = 0.219$) for cortisol concentrations.

There were no differences ($P > 0.05$) in any leukocyte measures between treatments, but there were differences ($P < 0.05$) between the periods of gestation at 0, 30, 60, and 90 days in white blood cell (WBC) counts, number and percent of

neutrophils, number and percent of eosinophils, percent lymphocytes, and the N:L ratio (Table 1). There was no interaction between treatment and period ($P > 0.05$) for any leukocyte measures.

There were no differences between treatments for percent of neutrophils positive for OB ($P = 0.58$), CON: 59.1 ± 4.54 %; ETOH: 55.5 ± 4.54 %, or for the florescence intensity response of neutrophils positive for OB ($P = 0.95$), CON: 164 ± 30.2 GMFI; ETOH: 162 ± 30.2 GMFI. There was a difference ($P < 0.050$) between the different periods of gestation at 0, 30, 60 and 90 days, of both percentage positive for OB (Figure 6) and florescence intensity response (Figure 7). There was no interaction between treatment and period ($P > 0.05$) for either OB measure.

Reproductive performance

There were no differences ($P > 0.05$) between sow weights before the study started (CON: 179 ± 8.09 kg; ETOH: 186 ± 8.09 kg), how much weight was gained during gestation (CON: 35.0 ± 5.95 kg; ETOH: 44.1 ± 5.95 kg), or sow weight after lactation (CON: 196 ± 7.04 kg; ETOH: 199 ± 7.04 kg). Sows given ethanol throughout gestation weighed more ($P = 0.04$) five days before farrowing (CON: 214 ± 4.93 kg; ETOH: 230 ± 4.93 kg), and tended to lose more ($P = 0.101$) weight during the lactation period (CON: -18.4 ± 4.98 kg; ETOH: -30.8 ± 4.98 kg).

There were no differences ($P > 0.05$) in the number of pigs per litter for total of pigs born (CON: 14.1 ± 0.784; ETOH: 13.5 ± 0.784), pigs born alive (CON: 14.1 ± 0.854; ETOH: 13.1 ± 0.854), mummies (CON: 0.13 ± 0.125; ETOH: 0.13 ± 0.125),

stillborns (CON: 0.00 ± 0.125; ETOH: 0.25 ± 0.125) or total pigs born dead (CON: 0.13 ± 0.206; ETOH: 0.38 ± 0.206).

There were no differences ($P > 0.05$) in the litter birth weight (CON: 1.69 ± 0.086 kg; ETOH: 1.84 ± 0.086 kg), percent of piglet mortality (CON: 17.0 ± 5.01; ETOH: 17.5 ± 5.01), number of pigs weaned per litter (CON: 11.3 ± 0.78: ETOH: 10.9 ± 0.78), or the litter wean weight (CON: 6.51 ± 0.292 kg; ETOH: 6.57 ± 0.292 kg).

Offspring

Weight

There were no differences ($P > 0.05$) between treatments for birth weight (CON: 1.66 ± 0.112 kg; ETOH: 1.87 ± 0.112 kg), wean weight (CON: 6.14 ± 0.478 kg; ETOH: 6.93 ± 0.478 kg), average daily gain during the lactation period (CON: 0.21 ± 0.019 kg; ETOH: 0.24 ± 0.019 kg) or at 180 d of age (market) weight (CON 114 ± 2.14 kg; ETOH 117 ± 2.14 kg).

Immune response

There were no differences ($P > 0.05$) between treatments for any leukocyte count measured at one week of age; white blood cell count: CON: 1253 ± 157.6; ETOH 1326 ± 157.6, number of neutrophils: CON: 691 ± 104.3; ETOH: 713 ± 104.3, number of lymphocytes: CON: 475 ± 44.2; ETOH: 517 ± 44.2, number of monocytes: CON: 79.7 ± 15.67; ETOH: 87.0 ± 15.67, number of eosinophils: CON: 8.00 ± 1.965;

ETOH: 8.37 ± 1.965, number of basophils: CON: 5.41 ± 3.87; ETOH: 12.1 ± 3.87, percentage of neutrophils: CON: 539 ± 23.7; ETOH: 530 ± 23.7, percentage of lymphocytes: CON: 394 ± 25.2; ETOH: 399 ± 25.2, neutrophil to lymphocyte ratio: CON 1.48 ± 0.152; ETOH: 1.39 ± 0.152, percentage of monocytes: CON: 61.5 ± 5.08; ETOH: 65.0 ± 5.08, percentage of eosinophils: CON: 6.29 ± 1.179; ETOH: 6.14 ± 1.179, or percentage of basophils: CON: 5.29 ± 3.130; ETOH: 9.15 ± 3.130.

At weaning, ETOH piglets had lower ($P = 0.034$) percent eosinophils and tended to have lower ($P = 0.078$) eosinophil numbers than CON piglets (Table 2). There were differences ($P < 0.050$) before, and 24 hours after weaning, for the number of neutrophils, lymphocytes, monocytes, eosinophils, and for the percent of neutrophils, lymphocytes, eosinophils and the neutrophil to lymphocyte ratio (Table 3). There was a tendency ($P = 0.092$) for a greater percent of monocytes before weaning compared to 24 hours after. There was no interaction between treatment and time for all measures except for a tendency ($P = 0.094$) in the percent of eosinophils. Control and ETOH pigs were different at baseline but not 24 hours after weaning, and differed between baseline (0 h) and 24 hours after weaning within each treatment.

Ethanol pigs had a greater ($P = 0.014$) percent of neutrophils positive for OB compared to CON pigs (CON: 29.4 ± 1.94 %; ETOH: 40.9 ± 1.94 %) at one week of age, but there were no differences ($P = 0.93$) in the florescence intensity response of neutrophils positive for OB (CON: 128 ± 26.8 GMFI; ETOH: 132 ± 26.8 GMFI).

Stress vocalizations during processing

There were no differences ($P > 0.05$) of stress vocalizations between CON and ETOH piglets at three days of age during processing (Table 3). Boars displayed more stress vocalizations than gilts during teeth clipping ($P = 0.001$), tail docking ($P = 0.002$) and for the entire process ($P < 0.0001$) (Table 3; Figure 8). There was no interaction ($P > 0.05$) between treatments and sex for any processing procedure.

Behavioral tests

Experiment 1

Backtest

There were no differences ($P > 0.05$) between CON and ETOH piglets in vocalization duration (CON: 16.5 ± 3.23 sec; ETOH: 11.9 ± 3.23 sec), vocalization frequency (CON: 2.07 ± 0.324 No.; ETOH: 1.65 ± 0.324 No.), or in escape behavior duration (CON: 13.3 ± 2.058 sec; ETOH: 9.99 ± 2.058 sec) or frequency (CON: 2.62 ± 0.341 No.; ETOH: 2.01 ± 0.341 No.) during the backtest.

There was a difference between CON and ETOH pigs categorized into low, intermediate, and high responders in the vocal response ($P = 0.011$; Chi Sq = 9.02, 2df) (Table 4) and in the escape response ($P = 0.0001$; Chi Sq = 18.07, 2 df) (Table 5).

Isolation test

There were no differences ($P > 0.05$) between CON and ETOH pigs in the frequency of behaviors in the two hour isolation test for number of bouts standing (CON: 197 ± 62.3 No.; ETOH: 138 ± 62.3 No.), inactive (CON 240 ± 63.7 No.; ETOH: 176 ± 63.7 No.); active (CON: 178 ± 55.4 No.; ETOH: 108 ± 55.4 No.),

vocalizing (CON: 42.0 ± 22.04 No.; ETOH: 20.8 ± 22.04 No.), or escape attempts (CON: 72.6 ± 14.91 No.; ETOH: 55.0 ± 14.91 No.). Control pigs spent more (P = 0.019) time active in the isolation test than ETOH pigs, but there were no differences ($P > 0.05$) in the duration of any other behaviors (Table 6).

Open field test

There were no differences ($P > 0.05$) between CON and ETOH pigs in the duration of time spent in each square or locomotor bout in the open field test (Table 7), except for time spent standing ($P = 0.028$), and time spent active ($P = 0.061$). Pigs spent more time ($P < 0.0001$) in the square where the novel object was placed during the novel period than the familiarization period, and spent more time ($P = 0.010$) in the square they entered the arena in during the familiarization period compared to the novel period. Pigs were also more active ($P = 0.032$) during the familiarization period compared to the novel period (Table 7). There was no interaction between treatment and period ($P > 0.05$) for the time spent in different areas of or performing different behaviors in the open field test, but there was an interaction tendency ($P = 0.091$) for time spent in the square 1.

There were no other differences ($P > 0.05$) between treatments in the frequency of behaviors or number of times entered a specific square in the open field test. There were many differences ($P < 0.05$) in the number of times pigs entered squares 1, 2, 3, 4, 6, 7, 8, and 9 (Table 8) between the familiarization and novel periods. There was a tendency for pigs to stand more times ($P = 0.093$) and display

more inactive bouts ($P = 0.083$) during the familiarization period compared to the novel period. There was no interaction between treatment and period ($P > 0.05$) for the frequency of behaviors in the open field test.

During the novel period there were no differences ($P > 0.05$) in the number of interactions with the novel object (CON: 5.88 ± 0.558; ETOH: 6.45 ± 0.558), the time spent interacting with the novel object (CON: 66.9 ± 15.24 sec; ETOH: 68.4 ± 15.24 sec) or the latency to interact with the novel object (CON: 234 ± 47.0 sec; ETOH: 276 ± 47.0 sec) between CON and ETOH treatments.

Social status

There were no differences ($P > 0.05$) between CON and ETOH pigs in teat order (CON: 2.07 ± 0.113; ETOH: 1.90 ± 0.113) or social test rank (CON: 1.61 ± 0.123; ETOH: 1.39 ± 0.123). Pigs exposed to ethanol prenatally were submissive ($P = 0.02$) compared to CON pigs at eight weeks of age in finishing (CON: 1.84 ± 0.280; ETOH: 2.88 ± 0.280). Chi square analysis revealed no differences ($P > 0.05$) in teat order or social test rank, but also revealed ETOH pigs to be submissive compared to CON pigs at finishing age ($P = 0.002$; Chi sq = 14.96, 3df) (Table 9).

There were no differences ($P > 0.05$) between CON and ETOH pigs in the cortisol response to the social test (CON: 32.2 ± 2.49 ng/mL; ETOH: 35.9 ± 2.49 ng/mL), between pigs classified afterwards as dominant or submissive (DOM: 32.5 ± 5.47 ng/mL; ETOH: 35.6 ± 5.47 ng/mL), or the time before or after the test (BEF: 30.8

± 2.79 ng/mL; AFT: 37.3 ± 2.79 ng/mL). There was no interaction between treatment and time (P =0.86) or between social status and time (P = 0.50) for the social test.

Mazes

There were no treatment or repetition (rep) differences (P > 0.05) in the duration of locomotor and learning/cognition behaviors in Maze I (Table 10) or Maze I Memory (Table 11) and no differences between treatments in Maze II (Table 12). In Maze II the trial repetition was significantly different for time spent active (P = 0.044), time on the right track (P = 0.051), the total time to reach the reward (P =0. 017), and the latency to be near the reward but not eat it (P = 0.007). By the third and fourth repetition, pigs were less active, and were completing the maze more quickly compared to the first and sometimes second trial. There was an interaction between treatment and maze repetition for the amount of time spent standing (P = 0.039; Figure 8) in Maze I, time spent active (P = 0.09) and time spent on the right track (P = 0.018) in Maze I Memory (Figure 9), but none (P > 0.05) were found in Maze II.

The number of times pigs displayed locomotor behaviors, made wrong turns, came in proximity of the reward, or the number of pigs that completed the maze did not differ (P > 0.05) between CON and ETOH pigs in Maze I, Maze I Memory, or Maze II. In Maze I (Table 13), pigs stood more (P = 0.043) in the first repetition compared to the second and fourth. The third and fourth trial had fewer (P = 0.044) number of wrong turns compared to the first trial, which also tended to have more errors compared to the second. There was also a tendency (P = 0.06) for trial

repetitions to differ for the number of active and inactive bouts. No differences ($P > 0.05$) were found across repetitions in Maze I Memory (Table 14), but repetitions did differ ($P = 0.029$) in the number of wrong turns in Maze II (Table 15) with fewer errors in the third and fourth repetitions compared to the first trial, and a tendency for even the second repetition to have fewer errors compared to the first. The number of times in proximity to the reward was significant ($P = 0.053$) for the interaction between treatment and repetition, with ETOH pigs during the third repetition getting close to the reward more times than CON pigs (Figure 10). The interaction for the number of pigs that completed the maze was significant ($P = 0.038$) between treatment and maze repetition in Maze I Memory. Control pigs completed the maze fewer times in the second repetition with ETOH pigs completing more the second time (Figure 11). There were no ($P > 0.05$) interactions between treatment and repetition for the number of any behaviors, wrong turns, number of times near the reward or completed the maze in Maze II.

Experiment 2

Backtest

There were no differences ($P > 0.05$) in the gestation treatment, lactation treatment, or the interaction between gestation and lactation treatments for the number and duration of vocalizations or escape attempts during the backtest (Table 16). There was no difference ($P = 0.43$) between the four treatments in coping response style based on vocalizations (Table 17), but when response category was determined by

escape attempts, (Table 18) EE then CC pigs had the most HR with CC also having the most intermediate responders ($P = 0.001$; Chi square = 22.39, 6 df).

Isolation test

The time pigs spent in different locomotor positions and displaying stress behaviors in the two hour isolation test (Table 19) tended ($P = 0.07$) to be different for gestation treatment with CON pigs more active compared to ETOH pigs. There were no other behavioral differences ($P > 0.05$) for the gestation treatment. Pigs that were reared on ETOH sows, regardless of gestation treatment, tended to spend more time standing compared to pigs that lactated on CON sows ($P = 0.08$), but no other differences were found in the lactation treatment. There was a tendency for an interaction between gestation and lactation treatments ($P = 0.08$) for the time pigs spent standing. Pigs in the CE treatment stood longer compared to CC, EC, and EE pigs (CC: 1817 ± 625.6 sec; CE: 4089 ± 625.6 sec; EC: 1963 ± 625.6 sec; EE: 1983 ± 625.6 sec).

Similar to the duration of behaviors in the isolation test, CON gestated pigs also tended ($P = 0.07$) to display more active behavior bouts compared to ETOH pigs. No other differences were found for the number of locomotor and stress behaviors for gestation treatment or for lactation treatment (Table 20). There were tendencies between gestation and lactation treatments for the number of times stood ($P = 0.08$) and for number of times active ($P = 0.09$). Pigs from CON sows but reared on ETOH sows stood more times compared to EE pigs, but no other treatments differed from

each other (CC: 77.8 ± 18.23 No.; CE: 115 ± 18.23 No.; EC: 79.5 ± 18.23 No.; EE: 59.5 ± 18.23 No.). Pigs from CON sows that were reared on ETOH sows also displayed more active behaviors compared to all other treatments (CC: 69.8 ± 73.95 No.; CE: 124 ± 73.95 No.; EC: 53.0 ± 73.95 No.; EE: 49 ± 73.95 No.).

Open field test

The time pigs spent in each of the nine squares in the open field test and performing locomotor behaviors was assessed for fixed (gestation, lactation, and period) effects and interactions (gestation*lactation, gestation*period, lactation*period, and gestation*lactation*period) (Table 21). Pigs from ETOH gestating sows tended ($P = 0.08$) to spend more time in square 7 where they entered and exited the arena, and pigs from CON gestating sows spent more time ($P = 0.044$) in square 8. There were no other differences ($P > 0.05$) for gestation treatment or lactation treatment in the open field test. Pigs, regardless of treatment, spent more time ($P = 0.004$) in square 6 where the novel object was placed during the novel period compared to the familiarization period, and spent more time ($P = 0.001$) in square 7 where they entered the arena during the familiarization period. Pigs tended ($P = 0.08$) to spend more time being active in the familiarization period compared to the novel period. There was no interaction between gestation treatment and period of the test ($P > 0.05$) for the duration in any square or behaviors. There was a tendency ($P = 0.07$) for pigs reared on CON sows to spend less time in square 3 during the novel period (CON-Familiar: 39.1 ± 6.53 sec; CON-Novel: 88.0 ± 6.53 sec; ETOH-Familiar: 27.4 ±

6.53 sec; ETOH-Novel: 20.8 ± 6.53 sec), but no other interactions between lactation treatment and period were observed ($P > 0.05$). There was a tendency ($P = 0.10$) for EC pigs to spend more time in square 4 during the familiarization period compared to CC and EE pigs; in the novel period there was a tendency for CC pigs to spend more time in square 4 compared to EE pigs, but only EC pigs differed between the amount of time spent in the familiar and novel within the treatment (CC-Familiar: 21.0 ± 11.10 sec; CC-Novel: 34.1 ± 11.75 sec; CE-Familiar: 43.9 ± 11.10 sec; CE-Novel: 15.5 ± 11.75 sec; EC-Familiar: 50.2 ± 11.10 sec; EC-Novel: 14.1 ± 11.75 sec; EE-Familiar: 18.8 ± 11.10 sec; EE-Novel: 2.69 ± 11.75 sec). There was also a tendency ($P = 0.07$) for EC pigs to spend more time in square 7 where they entered the arena in the familiarization period compared to all other treatments in the familiarization period. There were no differences across treatments in the novel period, but both EC and EE pigs spent more time in square 7 in the familiarization period compared novel period (Figure 12). There were no other three way interactions between gestation treatment, lactation treatment and period ($P > 0.05$) for any other squares or behaviors in the open field test.

The frequency of times pigs entered each square and displayed locomotor behavior bouts in the open field are presented in Table 22. Pigs from the CON gestation treatment displayed a greater ($P = 0.036$) frequency of active bouts compared to ETOH gestation treatment pigs. No differences ($P > 0.05$) were found for any squares or other behaviors between gestation treatments, and none were found between CON and ETOH lactation treatments. Pigs, regardless of gestation or

lactation treatment, crossed into all squares except square 5 and 6 more ($P < 0.05$) times in the familiarization period compared to the novel period. There was a tendency for an interaction between gestation and lactation treatments for the number of times entering into the middle of the arena ($P = 0.08$) and for active bouts ($P = 0.10$). Control pigs that stayed with their birth mother (CC) entered the middle of the arena more times compared to the CE pigs (Figure 13), while CE pigs displayed more active bouts compared to EE pigs (Figure 14).

There were no differences ($P > 0.05$) between gestation treatments, lactation treatments or in interactions between gestation and lactation treatments with a novel object for duration of interactions (CC: 65.2 ± 22.72 sec; CE: 42.0 ± 22.72 sec; EC: 49.8 ± 22.72 sec; EE: 67.5 ± 22.72 sec), number of interactions (CC: 6.08 ± 1.28 No., CE: 6.88 ± 1.28 No.; EC: 6.25 ± 1.28 No.; EE: 6.56 ± 1.28 No.), or the latency to interact with the novel object (CC: 198 ± 13.1 sec; CE: 195 ± 13.1 sec; EC: 208 ± 13.1 sec; EE: 193 ± 13.1 sec).

Social status

At all three times social status was assessed, there was no difference ($P > 0.05$) in the behavioral response between pigs in lactation treatment, and no interactions between gestation and lactation treatments. Comparing only the gestation treatment, ETOH pigs were dominant compared to CON pigs in the teat order ($P = 0.015$) (CON: 2.40 ± 0.150 rank; ETOH: 1.80 ± 0.150 rank), and when socialized with a weight matched, unfamiliar pig ($P = 0.027$) (CON: 1.78 ± 0.143 rank; ETOH: 1.25 ± 0.143

rank). However, in the finishing phase ($P = 0.028$) CON pigs were dominant over ETOH pigs (CON: 1.80 ± 0.327 rank; ETOH: 2.98 ± 0.327 rank).

Chi square analysis showed a trend ($P = 0.09$) for EE pigs to have the cranial teats, EC pigs were intermediately ranked, and CC and CE pigs were the only treatments with caudal teats in teat order social status (Table 23). In the social interaction test (Table 24), more ($P = 0.05$) EC and EE pigs were dominant overall compared to CC and CE, which were more submissive. However, again in the finishing phase (Table 25), CC and CE pigs were ranked as dominant ($P = 0.03$) with EC and EE in intermediate and submissive ranks.

There were no differences ($P > 0.05$) between CON and ETOH pigs in cortisol concentrations during the social interaction test for gestation treatment (CON: 40.2 ± 7.18 ng/mL; ETOH: 53.7 ± 7.18 ng/mL), or in lactation treatment (CON: 45.4 ± 6.95 ng/mL; ETOH: 48.5 ± 6.95 ng/mL). There was no interaction between gestation and lactation treatments ($P > 0.05$) for social interaction cortisol concentrations before and after the test.

Mazes

The time spent performing locomotor and cognitive behaviors in Maze I are found in Table 26. There were no differences ($P > 0.05$) for gestation treatment, lactation treatment, or trail repetition in Maze I, except for latency to come in proximity of the reward ($P = 0.025$). Maze I, repetition (rep) 2 was quicker compared to reps 1 and 3, and tended to be quicker than rep 4. There were no two-way or three-

way interactions ($P > 0.05$) for the duration spent performing behaviors or completing Maze I. For the number of behavior bouts (Table 27) in Maze I, there were no gestation treatment, lactation treatment, or trial rep differences ($P > 0.05$). There was a tendency between gestation and lactation treatments to display more active bouts ($P = 0.06$), more standing bouts ($P = 0.07$), and more inactive bouts ($P = 0.06$). Ethanol-Control pigs tended to display fewer active bouts (CC: 12.7 ± 1.006 No.; CE: 11.4 ± 1.006 No., EC: 9.07 ± 1.006 No.; EE: 12.8 ± 1.006 No.), and stood fewer times (CC: 11.5 ± 1.003 No.; CE: 10.2 ± 1.003 No.; EC: 7.90 ± 1.003 No.; EE: 11.5 ± 1.003 No.) compared to CC and EE pigs, but were not different from CE pigs. For the number of inactive bouts, EC pigs were inactive fewer times compared to EE pigs, and tended to display fewer inactive bouts compared to CC pigs, but were not different from CE pigs (CC: 11.6 ± 1.057 No.; CE: 10.2 ± 1.057 No.; EC: 7.89 ± 1.057 No.; EE: 11.9 ± 1.057 No.).

In Maze I Memory (Table 28), CON gestation pigs tended to spend more time ($P = 0.07$) standing compared to ETOH pigs, and pigs reared on CON sows tended to be quicker ($P = 0.06$) to get close to the reward than pigs reared on ETOH sows. There was a tendency for an interaction between gestation and lactation treatments ($P = 0.06$) for the latency to get in proximity to the reward. Pigs birthed from CON sows but reared on ETOH sows were quicker to get close to the reward compared to all other treatments (Figure 15). There were no differences ($P > 0.05$) for any other fixed effects, two-way or three-way interactions for Maze I Memory. The frequency of behaviors for Maze I Memory can be found in Table 29. There was a tendency ($P =$

0.08) for pigs reared on ETOH sows regardless of gestation treatment to make more wrong turns compared to CON lactated pigs. No other significances ($P > 0.05$) were observed for gestation treatment, lactation treatment, trial rep, or any two way or three way interactions.

For gestation treatment, CON pigs spent more time ($P = 0.026$) active, and tended to spend more time ($P = 0.09$) on the right track to the reward compared to ETOH pigs in Maze II (Table 30). For fixed effects, there were no other gestation treatment, lactation treatment, or trail repetition differences ($P > 0.05$) for Maze II. There was a trend for an interaction between gestation and lactation treatments ($P = 0.08$) for the time spent inactive, CE pigs spent the most time inactive but there we no differences between treatments (Figure 16). There were no more ($P > 0.05$) two way or three way interactions for Maze II. The frequencies of behaviors for Maze II are found on Table 31. Gestation CON pigs displayed more active ($P = 0.051$) bouts, and tended to display more standing ($P = 0.08$) and more inactive ($P = 0.10$) bouts compared to ETOH gestation pigs. More gestation ETOH pigs completed Maze II successfully compared to CON gestation pigs ($P = 0.030$). There was also a gestation by lactation treatment tendency ($P = 0.09$) for EE pigs to complete the maze significantly more compared to CC and CE pigs, and tended to more than EC pigs (Figure 17). No other differences ($P > 0.05$) for the frequency of behaviors in Maze II were observed.

Drinking behavior

When adolescent equivalent pigs were housed individually to assess drinking behavior, EE pigs drank more ($P = 0.030$) 20% ethanol than CE and EC pigs but not more than CC pigs (Figure 18). However, when pigs were left in their home pens and allowed to drink with access to pen mates, there were no differences ($P > 0.05$) in the amount of 20% ethanol drank, the number of drinking bouts, or time spent drinking (Table 32) between gestation treatment, lactation treatment, or an interaction between gestation and lactation treatments.

A list of all significant gestation main effects are provided in a summary table (Table 33).

Discussion

Sows

Drinking

In the present study, sows were given 20% ethanol free choice throughout the entire length of gestation. The first10 weeks of gestation sows drank 2 g/kg BW or more ethanol when consumption across animals was averaged. However, after 10 weeks of gestation ethanol consumption decreased. The lowest daily intake of any individual sow was 0.17 g/kg and the highest 6.17 g/kg, which indicated there was large variation within an individual animal's daily intake and across animals. Tumbleson et al. (1981b) found Sinclair (S-1) miniature sows were more variable in daily ethanol intake compared toS-1 males, and intake varied with the estrus cycle. Therefore, hormone fluctuations from pregnancy could have also influenced daily ethanol consumption.

An average of 2 g/kg or higher of ethanol consumption should be enough to produce intoxicated BEC levels. Wistar rat dams consumed 2.83 to 3.97 g/kg of ethanol daily ranging in the low and medium range of BECs, but at levels assumed to be produced by moderate ethanol consumption in humans (Barbaccia et al., 2007). This, however, depended on the time between drinks and whether or not drinks were taken with a meal (Eckardt et al., 1998). It is unknown exactly when the animals in this study drank, but typical liquid consumption in sows is early in the day and after meals. If sows drank after a meal, then ethanol absorption could be skewed due to contents in the stomach, and could be one reason high BECs were never observed in this study.

When rodents were first exposed to a drug, odor and taste were used to assess the solution as attractive, aversive, or indifferent (Wolffgramm and Heyne, 1995). The first few days of intake, animals consumed high doses, and then slowly reduced their intake overtime until only low levels were consumed (Wolffgramm and Heyne, 1995; Wolffgramm, 1990; Wolffgramm and Heyne, 1991). Social housing conditions could also affect daily consumption. Wolffgramm and Heyne (1991) found the highest quantity of ethanol (2.05 g/kg/day) consumed was in rats isolated long-term, with no differences in group housed (1.47 g/kg/day) and contact-caged (1.57 g/kg/day) rats, and isolated rats had a preference for higher percent ethanol solutions than group housed rats. The sows were individually housed with minimal contact to adjacent animals. Therefore, the social environment may be one reason sows did drink the first two months of gestation. Although variability was high between free-choice rats,

individual rats maintained a relatively stable level of consumption when the environment did not change.

The most influential factors that lead to birth defects of PAE are the amount of ethanol that reaches the fetus, and the dose, rate of consumption, and pattern of exposure (Riley and McGee, 2005; Thackray and Tifft, 2001). Timing of ethanol exposure to the fetus determines which structures are affected. A free-choice model does not control for the time of intake relative to the stage of fetal development. However, Korkman and coworkers (1998) observed children who were exposed to ethanol throughout the entire length of gestation had the most severe effects, so it was thought that no matter what period of gestation peak ethanol consumption occurred in this study, some developmental and/or behavioral effects should have been observed.

Peak consumption in the sows was the first 10 weeks of gestation, so development in both early- and mid-gestation could have been effected. Drinking during early gestation is thought to be the most sensitive period of exposure because of the gastrulation stages of embryogenesis and brain development (Sulik et al., 1984; O'Leary, 2004; Miller, 1996). In the second trimester, neuronal loss can occur from drinking (Barnes and Walker, 1981; Miller and Potempa, 1990), and more spontaneous abortions can result in women who were regular drinkers, drinking one or more drinks daily. Miscarriages that occurred in the second trimester of human pregnancies were more closely related to alcohol use than in the first trimester (Harlap and Shiono, 1980).

Health

The endocrine function and adrenal activity were changed by ethanol consumption, because ethanol and acetaldehyde increased the production and secretion of glucocorticoids (Coles et al., 1991). However, in the present study no effects of ethanol consumption on sow cortisol concentrations were observed. Cortisol concentrations have been found to be increased within 30 minutes of ethanol administration (Kinoshita et al., 2001), and in pregnant rats consuming ethanol increased basal levels and in response to a stressor (Levine and Mullins, 1966). Adrenal weights were also increased in response to chronic ethanol consumption during gestation in rats (Kakihana et al., 1968; Ellis, 1966; Weinberg and Bezio, 1987). Ethanol induced increases in plasma glucocorticoid concentrations were dependent on many factors, especially the dose of ethanol administered, type of ethanol administration, and the time to blood collection (Kakihana et al., 1968; Ellis, 1966; Weinberg, 1992; Ogilvie et al., 1997). Blood was taken for cortisol analysis at a specific time during gestation to account for the different periods of gestation, but blood samples may not have been taken close enough to the time after ethanol consumption to observe an increase in cortisol concentrations in this study.

There was a tendency for cortisol concentrations to be elevated at 60 days of gestation compared to day 0, the day before breeding. Glucocorticoids are required for fetal development, and gradually increase throughout gestation (Rosenthal et al., 1969). Therefore, it is not surprising that cortisol concentrations increased throughout gestation in sows. In normal human pregnancies, cortisol concentrations increased

from 149 ng/mL at 12 weeks to 352 ng/mL at 26 weeks of gestation then changed minimally until labor when concentrations significantly increased (Carr et al., 1981).

No differences were found between CON and ETOH sows in leukocyte counts or neutrophil function when measuring the immune response to drinking throughout gestation. Chronic alcohol abuse can lead to immunodeficiency (Cook, 1998; Roselle et al., 1993; Szabo, 1999), but sows in this study were not chronic heavy drinkers so it could be one reason immune differences were not observed. Alcoholism typically increased neutrophil numbers in the blood and in the liver and contributed to liver damage. But in late stage alcoholics, the number of neutrophils in the blood can be reduced leading to immunosuppression (Cook, 1998). Acute ethanol exposure has also been shown to impair phagocytic potential and decreased levels of neutrophils and macrophages in rats (Mufti et al., 1988), but differences between humans and rats have been observed (Bautista, 1997; Patel et al., 1996). Immune suppression also depends on ethanol concentrations, Mufti and coworkers (1988) found alveolar macrophage phagocytic activity was suppressed in rats, and the higher the concentration of ethanol rats consumed, the higher the degree of suppression even in acute models. Sows in the present study may have not consumed ethanol in excess to suppress their own health and immunity, but just enough to cross the placenta to affect the fetus.

Pregnancy itself is considered an immunosuppressive state, although it does not mean that pregnant women are necessarily more susceptible to infection (Sacks et al., 1999; Mori 1981). Sex steroids increased during pregnancy and can modify the

immune response. It is thought that these changes assist in preventing a maternal-fetal rejection response by decreasing the cell-mediated immune response (Grossman, 1985). The immune response changed over time of gestation regardless of treatment in this study. Total WBC counts, neutrophil number and percent, eosinophil number, and the N:L ratio decreased, while percentage of lymphocytes and monocytes increased by period of gestation. However, most human studies show that both neutrophil and lymphocyte counts increased with ethanol consumption. Leukocyte activation is seen during pregnancy; large numbers of macrophages, lymphocytes, monocytes, and granulocytes were found in maternal circulation (Sacks et al., 1999). This is consistent with findings from the this study for monocytes and lymphocytes, however granulocyte numbers (neutrophils and eosinophils) decreased with advanced pregnancy.

Contrary to these results, in normal human pregnancies as early as 3 weeks of gestation Luppi (2003) observed granulocytes that were significantly elevated compared to non-pregnant patients by mid-gestation, and found no differences in the percent of monocytes throughout pregnancy. In human patients, Kuhnert and coworkers (1998) found the total white cell counts were elevated as early as the first trimester then increased to even higher percentages later in pregnancy, this was attributed to an increase in granulocytes and decrease in lymphocytes. Differences among lab groups were found in lymphocyte number, with some showing no change (MacLean et al., 1992), while others have shown a decrease (Johnstone et al., 1994).

In New Zealand white rabbits, WBC counts were slightly but not significantly increased in early gestation, then decreased in late gestation, neutrophils and eosinophils decreased, and no differences were observed in basophils and monocytes (Kim et al., 2002). Kriesten et al. (1987) also found total leukocyte counts decreased in early-gestation of rabbits because of neutrophil, eosinophil, and basophil reductions, but found an increase in lymphocytes throughout gestation. Monocyte numbers were decreased in early-gestation then elevated during the equivalent to the second trimester in humans. Wells et al. (1999) found lymphocyte increased total WBC counts in early-gestation, but then declined due to decreases in neutrophil and lymphocyte counts. Eosinophil and basophils counts were low but minimally contributed to the decreased WBC count. Monocytes were increased 2-fold by mid-gestation. The majority of literature on human pregnancies showed an increase in neutrophils, opposite of some animal studies in rabbits and this study. This could be from elevated estrogen and glucocorticoids in humans (Pitkin and Witte, 1979) that may not effect neutrophils in rabbits (Kriesten et al., 1987) and by speculation, pigs. Differences between normal pregnant animals and humans suggest there are different physiological responses (Kim et al, 2002) among different species regarding leukocyte counts.

Not only does the number of monocytes and granulocytes typically increase, but similar to this study, so does phagocytic activity including oxidative burst (Shibuya et al., 1987; Barriga et al., 1994). Basal intracellular reactive oxygen species values were increased in granulocytes, monocytes, and lymphocytes, and oxidative burst showed an increasing trend during pregnancy (Sacks et al., 1998). Naccasha and

coworkers (2001) found phenotypic changes of the immune system during normal pregnancy were more marked in monocytes, while granulocytes showed more metabolic changes. In late-gestation, granulocytes have greater ability to respond to mediators by chemotaxis, oxidative burst, and up-regulation of adhesion molecules (Luppi et al., 2002). The increased granulocyte number and activity was thought to be beneficial to the pregnant woman to augment the defense mechanism against foreign substances (Barriga et al., 1994). However, the mechanism responsible for both phenotypic and metabolic changes is unknown (Naccasha et al., 2001). Innate or non-specific immunity activation was increased during pregnancy possibly to compensate for suppressed adaptive immunity. The monocytes then play a central role rather than the lymphocyte in immunological adaptation (Sacks et al., 1999; Shibuya et al., 1987).

Reproductive parameters

Sows that drank ethanol throughout gestation gained more weight compared to CON sows, which is contradictory of rodent studies. In rodent studies, ethanol groups weighed less and gained less weight during gestation (Bilitzke and Church, 1992; Weinberg et al., 1996; Osborn et al., 1998; Dursun et al., 2006) compared to control groups and/or pair-fed groups. However, these were at very high concentrations (6 - 12 g/kg). Even at low doses (2 - 4.3 g/kg) alcohol consuming dams weighed less in Wistar rats (Lee and Wakabayashi, 1985), and in Sprague-Dawley rats (Brocardo et al., 2012). No differences were found between ethanol and sucrose dams (Barbaccia et al., 2007; Ledig et al., 1990), and Allan et al. (2003) also observed no difference in

ethanol and control B6SJL/F1 maternal mice weights. However, the latter was over a 2 week span from start of the study until breeding, and they did not report maternal weights before and after parturition. Only one study reports that ETOH dams weighed slightly more than pair-fed dams, but not controls (Livy et al., 2003).

Reduced maternal body weight observed by others could be attributed to lower food consumption (Bilitzke and Church, 1992; Brocardo et al., 2012; Detering et al., 1979). Nutritional deficiencies were common in humans who drank during pregnancy, and chronic alcoholism interfered with nutrient ingestion, absorption and utilization (Thomson and Pratt, 1992). This relationship between ethanol and nutrition make some results hard to discern between a prenatal ethanol affect or from lack of proper nutrition during development. A decrease in food consumption was not observed in the present study, or differences between treatments. Barbaccia et al. (2007) also did not observe differences in fluid intake between ethanol and sucrose Wistar rat dams. Ethanol is more caloric than water, so the added calories from consuming the ethanol solution could be one explanation for increased weight observed during gestation in the present study. Glavas et al. (2001) found at mid- and late-gestation pair-fed Sprague-Dawley dams weighed less than ethanol dams, and Kim et al. (199) found the same during mid-gestation. Alcohol drinkers had a higher intake of calories compared to nondrinkers because of the calories in alcohol (Gruchow et al., 1985; Jones et al., 1982). In light drinkers, alcohol calories are additive, but in heavy drinkers, alcohol calories replace nonalcoholic calories (Gruchow et al., 1985). It can be speculated that the animals in this study were light drinkers, which is suggested by the variable and

low daily consumption, and therefore the calories from the ethanol solution were additive and caused them to gain more weight during gestation.

Using a moderate dose of ethanol (20% v/v) in a free-choice paradigm, no differences in the litter size, pigs born dead, or birth weights between ETOH and CON piglets were observed despite the increased ETOH sow weight. This is consistent with many studies that did not find differences in size and number of stillborns in rodent models (Glavas et al., 2001; Kim et al., 1999; Becker and Randall, 1989; Osborn et al., 1998), and only a few researchers have found a decreased number of live births or litter size (Weinberg, 1992; Murillo-Fuentes et al., 2001). These findings are more typical of FAS because spontaneous abortions are common (Jones and Smith, 1973; Harlap and Shiono, 1980). In this study, it was found that only ETOH sows had stillborn pigs, but this difference did not reach statistical significance, although it seems the teratogenic effects of ethanol did influence stillborns. Middaugh et al. (1988) did not observe differences in litter sizes, but dams whose diets were composed of 25% ethanol calories did have a reduced number of viable pups in the litter. It takes as little as drinking only twice a week to lead to spontaneous abortions in humans (Kline et al., 1980). Some investigators did find PAE pups weighed less than control and/or pair-fed pups (Glavas et al., 2001; Weinberg, 1992; Kim et al., 1999; Dursun et al., 2006), while others found no difference in birth weights (Weinberg, 1988; Becker and Randall, 1989; Barbaccia et al., 2007; Ledig et al., 1990; Allan et al., 2011) in comparison to this study.

In this study, a tendency for ETOH sows to lose more weight during the lactation period was found, but at weaning CON and ETOH sows did not differ in body weight. Offspring from ethanol sows numerically weighed more than CON offspring; however this difference was not statistically significant, and therefore the heavier weights of the fetuses was not the reason for increased weight loss during the lactation period. Detering et al. (1979) found ETOH and isoenergetic rat dams ate less food than control dams during the lactation period, and lost 30% of post-partum body weight, while controls increased their body weight 25%. Murillo-Fuentes et al. (2001) found maternal body weight decreased during lactation in the ethanol dams, despite the fact that the ethanol group had a higher total energy intake. The difference in that study compared to the present one was rats in the Murillo-Fuentes et al. (2001) study were given ethanol during lactation when sows were not. In both studies, the ETOH group lost weight during lactation regardless of feed intake. Sows given ethanol throughout gestation in the present study were not given ethanol during lactation. Another explanation of the lost weight could be that the extra calories from ethanol were no longer being consumed, which could have led to the decrease in weight. However, feed intake typically increases during lactation in order to increase milk production; therefore, it makes sense that an overall difference in body weight between the two treatments was not observed at weaning.

Offspring

Weight

In the present study, no differences in body weight at birth, weaning, or at adolescents in the offspring were found. As discussed above, differences in birth weight between PAE and CON animals conflict based on species and ethanol paradigm. Osborn et al. (1998) did not observe a difference in body weight of Sprague-Dawley rat pups at birth, but did find that ethanol and pair-fed pups weighed less than controls postnatal day 8 and at weaning. However, in adulthood when testing began, the difference in body weight no longer existed. Dursun et al. (2006) found ethanol exposed Wistar rat pups differed in weight at birth and one week later compared to control groups, but were not different by two weeks after birth. Becker and Randall (1989) also found no differences in pup weights at birth and 10 and 17 days of age, or at weaning. However, a few studies have shown that pups weights were not different early in life, but beginning at weaning throughout adulthood ethanol C57BL/6c mice (Middaugh et al., 1988) and Long-Evans rats (Vorhees, 1989) weighed less. Middaugh et al. (1988) speculated that the PAE offspring were not physically mature enough to interact in behaviors required for food regulation.

It is known that alcohol consumption can effect milk production and let down (Mennella, 2001), but this may have a greater influence if consumption occured during lactation or previous to a nursing bout. Murillo-Fuentes and coworkers (2001) observed a reduction in milk consumption and suckling behavior in pups, but dams did consume ethanol during the lactation period. Offspring in Vorhees' (1989) study were exposed both prenatally and postnatally to ethanol, so the differences in weight at

older ages cannot be separated between the prenatal and postnatal effects of ethanol. The present study did not quantify nursing behavior, but no differences in frequency of nursing bouts or suckling behavioral problems were observed. Offspring from ethanol consuming dams may be able to "catch up" on body weight if there were an initial difference, and may not lose weight later in life if proper maternal care was given to the offspring during the suckling phase (Allan et al., 2003), and dams were nursing the offspring frequently enough to make up for any reduction in milk production.

Health

Prenatal exposure to ethanol can affect offspring immunity and increase disease susceptibility. Depressed lymphocyte counts, lymphocyte proliferation, and decreased immunoglobulins (Gottesfeld and Abel, 1991; Zhang et al., 2005; Johnson et al., 1981) have been found in the adaptive immune response. At one week of age, no difference in lymphocyte counts or percentages were found, but the present study did not measure the functionality of the adaptive immune system. Johnson et al. (1981) found a reduction in total circulating lymphocytes. This decrease could be from the growth retardation in FAS children because both FAS children and children that were small for their gestational age had lower counts than control children, and FAS children had the lowest count. Under-nutrition can influence the immune system suppressing the host's defense mechanisms (Weinberg and Jerrells, 1991), however, there was no difference in offspring weights and intrauterine growth between CON

and ETOH pigs in this study. Therefore, the lack of nutritional effect could be one reason differences in lymphocyte counts were not observed at one week of age.

The innate immune system was more variable in response to prenatal ethanol exposure, and deficits were not usually observed in animal studies (Zhang et al., 2005). Some studies found low counts of eosinophils and neutrophils (Gottesfeld and Abel, 1991), decreased phagocytosis (Gautheier et al., 2005; Ping et al., 2007), while others had not found differences in specific measures (Grossmann et al., 1993; Johnson et al., 1981). The present study observed no differences in total leukocyte counts, neutrophil, eosinophil, or basophil counts or percentages at one week of age. These findings were comparable to Grossmann et al. (1993) who observed no differences in WBC counts, T and B lymphocytes, or monocytes between ethanol and control pigtailed macaques offspring, and Johnson et al. (1981) who found neutrophil counts were not different in FAS children. At weaning in this study, the percent of eosinophils were reduced in ETOH pigs compared to CON pigs. Basal levels in the immune system were usually normal in PAE animals, and it was not until stressed conditions that deficits were seen (Giberson and Weinberg, 1995; Zhang et al., 2005).

Prenatally alcohol exposed piglets had a greater percentage of neutrophils positive for oxidative burst compared to CON piglets at baseline, but no differences existed between the florescence intensity of neutrophils positive for OB. Macrophage phagocytosis per cell was decreased 30% in prenatally ethanol exposed guinea pigs, and reduced the percentage of cells positive for *Staphylococcus aureus* ingestion (Gauthier et al., 2005). Alveolar macrophages also were affected by PAE.

Phagocytosis was dysfunctional and unable to internalize bacteria, and macrophage apoptosis increased (Ping et al., 2007), but the antioxidant S-adenosyl-methionine was able to protect alveolar macrophages from dysfunction caused by PAE. Other studies did not find differences in monocyte phagocytic function (Grossmann et al., 1993), or in neutrophil function to a nitroblue tetrazolium dye reduction assay (Johnson et al., 1981). No studies measured the oxidative burst capacities of peripheral blood neutrophils in response to *Escherichia coli* as in the present study, so direct comparisons across studies cannot be made. Because more neutrophils were positive for oxidative burst in ETOH pigs it could suggest that acute exposure to ethanol *in utero* may prime the offspring's innate immune response to pathogens.

Investigators have shown that the immune system is affected by both acute and chronic stress (Morrow-Tesch et al., 1994; Sutherland et al., 2006; Hicks et al., 1998; McGlone et al., 1993). Weaning is a stressful early life experience for pigs and can also affect the immune system. Niekamp et al. (2007) found weaning significantly influenced neutrophils, lymphocytes, monocytes, and other immune measures, but these measures were age-dependent suggesting the older the pig was at weaning the better its immune function. At weaning, regardless of prenatal treatment, elevated neutrophils and decreased lymphocytes, monocytes, and eosinophils were observed in the present study. Increased cortisol levels decreased the number of lymphocytes while it increased the number of neutrophils therefore, increased the N:L ratio (Salak-Johnson and McGlone, 2007 Hjarvard et al., 2009). Cortisol concentrations at weaning were not measured in the present study, but the increased N:L ratio could suggest

weaning induced a stressful state and altered the immune response of pigs 24 h after weaning.

Processing vocalizations

The routine management procedure of processing included tail docking, ear notching, teeth clipping, injections, and castration in males within the first few days of life. Piglets grunted more frequently when getting their tail docked compared to teeth clipped, ear notched, or when all three procedures are combined (Noonan et al., 1994), and both low and high-frequency calls accompanied handling and castration in piglets (Puppe et al., 2005). It is known these processes cause acute stress in piglets, but no differences in stress vocalizations between CON or ETOH piglets were observed when the procedures were broken down individually or combined. Children exposed prenatally to ethanol were fearless and had high pain tolerances which could make behavioral management difficult (Gardner, 2000).

Male pigs expressed a greater percentage of stress vocalizations for teeth clipping, tail docking and the total process compared to female pigs during processing. Typically men have a greater pain tolerance than women for mechanical pressure (Woodrow et al., 1972), cold pressure (Keogh et al., 2000), heat stimuli (Feine et al., 1991), and use different coping strategies. Males used sensory-focused coping while females used emotion-focused coping to decrease the affective pain experience (Keogh and Herdenfeldt, 2002). Even in neonates before learned reaction patterns, females expressed more facial features of pain during and after a capillary puncture

(Guinsburg et al., 2000). The percent stress vocalizations in the total process for male pigs could be higher because it included castration which is a painful procedure and caused vocalizations even when anesthetics or analgesics are applied (Sutherland et al., 2012; Sutherland et al., 2010). However, the individual behaviors of teeth clipping and tail docking which were performed before castration, were also higher in male pigs. Vocalizations however, may not be a suitable measure of pain though it is for stress.

In the open field test, females displayed lower stress responsivity than males because they were more active (Heinsbroek et al., 1988). Sex differences typically showed that females were less fearful based on fewer defecations and higher ambulation scores in the open field test, quicker to enter novel environments with increased exploration (Gray and Lalljee, 1974). Processing is a stressful event in a pig's life and may be a strong enough stressor to distinguish between sex differences.

Coping Strategy

Individual animals have diverse strategies for coping with their environment and stressors. These data support that high variability observed between individual animals in the same environment exists. No differences in the number or duration of vocalizations or escape attempts for the backtest in either experiment 1 or experiment 2 were found, but differences were seen when pigs were classified as Low, Intermediate, or High Responders. In Experiment 1, when the number of vocalizations was used to classify piglets, more CON piglets were classified as HR than ETOH pigs.

Number of escape attempts also showed more CON pigs as HR and IR. More individual ETOH pigs were classified as HR compared to other response categories in both vocalization and escape attempts, but still not as many as CON pigs, and more ETOH were LR compared to CON pigs as well. In experiment 2, categorizing coping styles based on vocalizations did not show any differences between treatments, but escape attempts showed EE pigs had the largest number of pigs in the HR category compared to the other three treatments. Control-Control pigs had the next highest number of HR with both EE and CC higher than CE and EC which did not differ from one another. Control-Control pigs also had more IR piglets than any other group.

High responders resisted the handler in the backtest and displayed a more active behavioral response and be classified as more aggressive, while LR had a passive coping style and were non-aggressive (Hessing et al., 1993). The type of coping strategy an animal uses reflects a predisposition to react in a predicted way to a challenging stimuli (Erhard and Mendl, 1999), and represents the animal's personality (Hayne and Gonyou, 2003), emotionality (Savage and Eysenck, 1964) or temperament (Grandin, 1993). Dysregulation of the HPA axis can cause greater anxiety in FASD, and make pigs more fearful, which can result in distinct reactions to fear, the fight/flight response or freezing.

In the first experiment, CON pigs were active responders and displayed a fight or flight response in stressful tests and showed more fear, while piglets whose mothers had ethanol were less active or froze. Prenatal ethanol consumption is known to affect the temperament of infants (O'Connor, 1996). A forced swim test is a measure of fear

or depression. Different strains of PAE rats showed increased immobility in a forced swim test compared to CON rats (Brocardo et al., 2012 Hellemans et al., 2010; Carneiro et al., 2005). The increase in immobility is a reflection of "behavioral despair," in which the animal "gives up" because escape is impossible (Porsolt et al., 1978; Carneiro et al., 2005), or could be passive coping mechanism to conserve energy (Borsini and Meli, 1988). Passive coping styles are characterized by a flexible, adaptable response that is externally driven and helpful in unfamiliar environments (Campbell et al., 2003; Ruis et al., 2000; Koolhaas et al., 1999). Hard et al. (1985) also found that the emotional reactivity in PAE rats was delayed by one to two days compared to controls, so could suggest differences between ETOH and CON treatments.

However, opposite results in the second experiment were found. Ethanol pigs that remained on their birth sow showed the greatest number of HR pigs compared to all other treatments, although CC had the second highest number and the most IR. Both groups of pigs that were not cross fostered were active responders (HR), but EE pigs had the most active responders. Stress from cross fostering in experiment 2 may have influenced the coping style response. Bilitzke and Church (1992) found untypical results in the forced swim test, PAE rats showed less immobility. Increased struggling in this test is an indication of more fearfulness, and less ability to calm down once excited. Active copers actively seek a way to remove themselves from the stressor (Koolhaas et al., 1999).

There are two coping strategies that exist within a population, both equally successful (Hessing et al., 1993), and both were found in PAE pigs across different experiments. Neither strategy is better than the other; they are simply different and may reflect how individual animals respond to stressors or behavioral challenges. Because coping strategy is partially determined by genetics, domestication of pigs may have affected individual characteristics through selective breeding of pigs that are more adapted to production conditions (Ruis et al., 2000).

Anxiety, Activity, Novelty

Isolation test

The social isolation test in this study was used as a measure of anxiety and stress in response to a psychological stressor. When animals that were typically group housed were isolated, physiologic and behavioral changes resulted (Heinrichs and Koob, 2005). In experiment 1, ETOH pigs spent less time being active in the two hour isolation test. In experiment 2, ETOH pigs in the gestation treatment tended to spend less time being active and displayed fewer active bouts than CON pigs similar to experiment 1, and ETOH pigs in the lactation treatment spent more time standing than CON pigs. The gestation and lactation treatment interaction showed CE pigs tended to spend more time standing compared to CC, EC or EE pigs, and displayed more standing bouts compared to EE pigs. But CE pigs also displayed more active bouts compared to all other treatments.

Isolation in pigs has been shown to cause high states of fearfulness and pigs became more responsive or vulnerable to environmental changes (Ruis et al., 2001a). Increased activity observed in CON and CE pigs suggested the ETOH piglets were less fearful and had a less active response to the isolation test. Bilitzke and Church (1992) found rats that were less immobile in the forced swim test were more fearful, and may reflect a more active and aggressive coping style to a psychological stressor. Ethanol increased HPA responsiveness to stress but this could be in a selective manner, as Taylor et al. (1982) found only cardiac puncture, noise and shake stressors and not novelty, environmental or metabolic stressors influenced stress induced HPA activity. So a psychological stressor of isolation may not have elicited hyperresponsiveness in the HPA axis, or the amount of ethanol pigs were exposed to *in utero* may not have caused HPA dysregulation.

Coping style may be another reason for the decreased anxiety during isolation. The passive response to stress as seen in more ethanol pigs may allow them to be more flexible and adaptable which is helpful in unfamiliar environments (Campbell et al., 2003; Ruis et al., 2000; Koolhaas et al., 1999). Control pigs were high responders to stress based on the backtest, although there were no differences between treatments in the time it took for pigs to “relax” or the duration of escape behaviors and vocalizations. High responder gilts were found to walk more than LR gilts the first hour the first day of an isolation test for (Ruis et al., 2001b). This activity is from actively trying to remove themselves from the stressor (Koolhaas et al., 1999). Correlations between coping strategy and behavioral tests were not made in this study,

however, in experiment 1 more CON pigs were HR, and even though in experiment 2 CE pigs had fewer HR than CC and EE treatments, it had more HR within the treatment. The fact that CE pigs both stood and displayed more active behaviors suggests they were more "restless" and made several postural changes. Repetitive, compulsive and aggressive behaviors resulted from prolonged isolation (Valzelli, 1973).

Isolation from the dam could also be one reason for differences in the isolation test. Pre-weanling rats showed arousal behaviors shortly after separation from the dam (Heinrichs adn Koob, 2005). Children prenatally exposed to alcohol showed a disorganized attachment style (Kelly et al., 2000) and often became self-reliant and unable to trust others because they could not rely on their caretaker (O'Connor, 1996). This characteristic of PAE offspring, could suggest the decreased activity and arousal observed in ETOH pigs was from a less sensitive attachment to the mother, or because they were stress resistant.

Open field test

The open field test can be used for many different behavioral measurements, such as anxiety, novelty seeking and activity. It is known that novelty can stimulate both fear and exploratory behaviors in animals, with fear decreasing with increasing experience with the stimulus (Montgomery, 1955). In both experiments, pigs spent more time in squares furthest from the entrance during the familiarization period. This period in the open field test was used to allow pigs to explore and investigate the novel

environment through rooting, nudging and sniffing (Taylor and Friend, 1986), so the time in multiple squares indicated they were exploring.

Pigs also spent more time in the area of the arena they entered the arena from, regardless of prenatal treatment in experiment 1, and ETOH pigs from the gestation treatment in experiment 2. Ethanol-Control pigs spent the most time there during the familiarization period compared to all other treatment groups in experiment 2 and compared to the novel period, and EE spent more time there during the familiarization than novel period. This could mean pigs were more fearful of the novel environment and froze when they were put in the arena, or that they spent more time in this area looking for an escape, with EC and then EE pigs being the most fearful of the environment in experiment 2. In experiment 1, CON pigs tried to escape more times in the novel period compared to ETOH pigs and also compared to the familiarization period. Control pigs also tended to spend more time in a corner away from the novel object during the novel period compared to ETOH pigs.

These data in experiment 1 indicate CON pigs were more fearful of being in a novel environment and also of a novel object. Osborn et al. (1998) found both male and female Sprague-Dawley control rats were less explorative than pair-fed or PAE rats, on day 1 of 2 in the elevated plus maze, and prenatally ethanol exposed males showed less fear and spent more time in open arms both days than controls. Hard et al. (1985) observed increased open field activity in PAE males but not females at 3 weeks of age. Carneiro et al. (2005) also found PAE rats were the ones to spend more time in open arms of the elevated plus maze. Prenatal ethanol exposure can have an anxiolytic

effect in rats, and if this is the case in experiment 1, CON pigs may seem more anxious in the novel environment and try to escape more than ETOH pigs.

In experiment 2, cross-fostered ethanol, EC, pigs were more fearful of the novel environment, and spent more time near the "exit" of the test. The elevated plus maze in rodent studies evaluated anxiety and was comparable to experiment 2, PAE rats showed more anxiety and fear by spending less time in open arms of the maze (Brocardo et al., 2012; Dursun et al., 2006). Osborn et al. (1998) found that exposure to an open field test prior to the elevated plus maze, reduced exploratory behavior in the PAE rats and in females more than males, and prenatally ethanol exposed females tended to show more fear on day 2, but males showed less fear. Overall activity in the open field test in experiment 2 showed that CON gestation treatment pigs displayed more active bouts, with CE pigs more than EE pigs. Decreased activity in EE pigs also may support that the ETOH gestation pigs were more fearful regardless if they were cross-fostered or not.

The open field test in rodent studies may be used as a better assessment of locomotion and activity because more test apparatuses are available for anxiety and stress. Children diagnosed with FAS are inattentive and hyperactive. Many have found rodents prenatally exposed to ethanol were more active in open field and elevated plus mazes (Ledig et al., 1990; Hellemans et al., 2010, Becker and Randall, 1989; Osborn et al., 1998), which were comparable to this study in that ETOH pigs tended to be more active than controls in the open field test in experiment 1, but in experiment 2 CE pigs were more active than EE pigs, but not from CC and EC treatments. The

increased activity in CE pigs was consistent with their reaction to the isolation test, so they may have displayed a more active fear response to the novel environment.

When animals experience fear during an exploratory situation, then exploratory behavior will greatly decrease (Montgomery and Monkman, 1955). Control pigs also tended to be less active in the open field test in experiment 1 compared to ETOH pigs also indicating they were more fearful of the novel environment, and displayed less active behavior. However, FAS children are known to be hyperactive and could explain the increase in activity seen in ETOH pigs. Contradictory, Fraser (1974) suggested that the continuous "restless" activity he saw in pigs in the open field test indicated fear of the test which could explain increased activity in CE pigs in experiment 2, supported by their increased activity and fear in the isolation test as well. Standing and inactivity bouts, regardless of prenatal treatment in experiment 1, were increased during the familiarization period, so just the novelty of being in a new environment may have elicited a fear response in all pigs.

There were no differences between treatments in the latency to interact with a novel object, duration, or number of interactions in either experiment. Therefore, the fear in the open field test did not keep any treatment from approaching and investigating the novel object. This is true if the strength of the fear response is small enough that allows the animal to begin exploration, and therefore diminish the initial fear (Montgomery, 1955). These results are opposite of what was hypothesized because FASD individuals were found by others to be impulsive and seek novelty and novel surroundings (Mattson et al., 1999; Davis et al., 2011; Nulman et al., 2004).

Prenatally ethanol exposed rats spent more time in novel environments than in familiar ones and showed less avoidance behavior to novelty (Ledig et al., 1990), and spent more time in the presence of a novel object (Allan et al., 2003).

Social status

Social behavior studies can prove difficult in humans because there is a lifespan of social experiences to account for, but even in rodent studies, social behavior have been found to be difficult because some of the same factors: genetics, dam-pup interactions, and social learning still influenced social behavior (Kelly et al., 2000). Fetal Alcohol Spectrum Disorder is characteristic of social impairments, which include socializing, interpersonal relationships, coping skills, play time, and aggressive behaviors (Mattson and Riley, 2000; Thomas et al., 1998; Davis et al., 2011). In experiment 1, no differences in social status were found during the lactation phase for either teat order or the social interaction test. However, during the finisher phase when pigs approached maturity, ETOH pigs were more submissive compared to CON pigs. In experiment 2, the gestation treatment showed ETOH pigs held more cranial teats and won more social interactions than CON pigs. Compared to experiment 1, at the equivalence to adolescence, CON pigs became dominant over ETOH pigs.

Prenatal ethanol exposure can disrupt suckling behavior, with altered nipple attachment behavior, abnormal sucking patterns, weak sucking and abnormal nipple shifting (Rockwood and Riley, 1990; Barron et al., 1991), so ETOH pigs

predominantly occupying caudal teats was expected. Establishing teat order is a measurement of dominance within the litter and not how efficient the pig is on that teat, although it can assumed if there was difficulty attaching and increased shifting then anterior teats could be easily lost to pigs that more efficiently suckle. Fewer ETOH pigs occupied lower teats but this was not statistically significant in experiment 1, but in experiment 2, EE pigs were the only treatment that occupied cranial/dominant teats and neither EE nor EC pigs occupied caudal teats. This suggested that EE pigs were more aggressive and were able to fight better for better teats. No EC pigs had the first teat but only occupied middle teats so they too may be more aggressive than CC and CE pigs, although middle teats may take longer to settle than the end teats on the udder (Fraser and Jones, 1975).

In the social interaction test, pigs were socialized with an unfamiliar pig for two hours. Temperament tests in FAS children showed kids were more willing to seek out strangers and approached the unfamiliar, a more socially aggressive personality trait (Nulman et al., 2004). When unfamiliar pigs were mixed they spent 17% of the first 90 min attacking one another (McGlone and Curtis, 1985). Dominance order in the social interaction test was determined by the pig that was more aggressive and initiated more attacks (McGlone, 1985). Prenatal ethanol exposure increased social investigation through sniffing, and wrestling and boxing in rats (Hamilton et al., 2010). In experiment 1, there were no significant differences however, the number of ETOH and CON pigs that were dominant or submissive in their fight were completely opposite of one another, with more ETOH pigs being dominant. This did reach

significance in experiment 2, where the ETOH gestation treatment pigs were dominant over CON, and all EC ranking as a dominant status. It is not surprising that pigs that occupied better teats also won more fights and were more dominant in the social interaction test. The teat order is highly correlated with social dominance rank, and the pigs that occupied anterior or dominant teats won more fight bouts and were socially dominant (Scheel et al., 1977).

At an early age in two different assessments of social status, ETOH pigs were more aggressive and therefore dominant over CON pigs. Aggressive behavior and more willingness to approach strangers are characteristic of prenatal ethanol exposure (Mattson and Riley, 2000; Nulman et al., 2004), and findings in pigs matched this description. In experiment 1, data may not have reached significance because novel environmental cues suppressed the social responsiveness of PAE animals (Lugo et al., 2003), and the socialization test was performed in a novel pen.

However, social deficits became more prominent with age (Kodituwakku, 2007), and FASD individuals fell further behind their peers in social skills (Thomas et al., 1998). Adolescence requires individuals to develop new social skills and changes in response to and interaction with stimuli in the environment can change (Spear, 2000). In this study, there was a shift in social status at adolescence, CON pigs in both experiments occupied dominant ranks over ETOH pigs. In experiment 2, no gestation ETOH treatment (EC or EE) pigs occupied a dominant rank, and EE pigs occupied every submissive rank except for in one group which was a CC pig. In humans, increased risk of peer rejection from poor social skills can lead FASD individuals to

become more aggressive, lead to anxiety and depression, antisocial behavior and addictive behaviors (Kully-Martens et al., 2012). These are also characteristic of passive-aggressive and antisocial personality disorders (Fryer et al., 2007). At adolescence when hormones are changing and social demands are complex, social deficits make it hard to establish and maintain relationships (Kupersmidt et al., 1990; Streissguth et al., 1996; Kodituwakku, 2007). Social recognition and communication were impaired in PAE rats (Kelly and Tran, 1997), which may contribute to the shift in social status observed in the present study at this time in development.

Mazes

Prenatal ethanol exposure altered learning and memory, and verbal, nonverbal and spatial learning. In humans, it is thought that deficits in learning had more to do with the acquisition of the material and not the ability to remember it over time (Mattson et al., 2001), meaning more repetition may be required to master the initial acquisition and encoding of new material (Kerns et al., 1997). These deficits were caused by hippocampal dysfunction and not from visual-motor or motivation deficits (Hamilton et al., 2003). Visual-spatial memory as a measure of cognitive function was a better assessment for learning and memory in animal models.

There were no significant differences for total maze duration between prenatal treatments in either experiment, but ETOH pigs were always quicker to complete the maze and spent less time in the maze. In experiment 1 maze I, ETOH pigs spent more time near the reward compared to CON pigs in the third replication. In experiment 1

maze I memory, more ETOH pigs completed the maze during the second replication compared to the first replication and compared to CON pigs. This was opposite of what the literature says about FASD. In a T maze, PAE rats required more trials to learn the new goal and made more reference and working memory errors (Riley et al., 1979; Zimmerberg et al., 1991). Sluyter et al. (2005) also found rats prenatally exposed to 35% ethanol were less likely to complete the maze and required more trials, but similar to this study, even at lower concentrations it took the PAE rats less time to complete the trial regardless of number of errors. Reyes et al. (1989) also found PAE rats completed the maze quicker even though they made more errors. Lee and Rabe (1999) found that PAE only showed a deficit in reversal learning but not acquisition learning in the T maze. No differences in acquisition of learning the mazes could mean that the task was easy to learn or PAE pigs learned just as well, if not better, than CON pigs.

The fact that PAE offspring may complete the maze faster could be from increased activity. In experiment 1 maze I, ETOH pigs tended to spend less time standing in the second replication in maze I compared to the first replication. In maze I memory, there was a tendency for ETOH pigs to spend more time in active behaviors during the first replication. In experiment 2 maze I, CC and EE pigs displayed more active, standing, and inactive bouts compared to EC pigs. In maze I memory, ETOH gestation treatment pigs spent less time standing than CON pigs. Increased activity could indicate pigs were quicker at getting to the reward which may or may not have been by chance. Reyes et al. (1989) observed that PAE rats moved more quickly and

with less purpose in a radial arm maze. Completing the maze could be by chance from increased activity, but more ETOH pigs successfully completed the maze in experiment 1 maze I memory and experiment 2 maze II compared to the number of completions made by CON pigs. Learning to solve new maze configures has been found to be linked to tonic immobility in rats, a measure of coping style. Poor performance in the maze was linked to active behavior responses and high performance to passive behavior responses (McGraw and Klemm, 1973). Prenatally ethanol exposed pigs displayed passive coping styles compared to CON pigs, which may allow for the flexibility and adaptability to response in an unfamiliar environment (Campbell et al., 2003; Ruis et al., 2000; Koolhaas et al., 1999), and to solve the mazes.

Different results were found in activity for experiment 2 maze II. Control gestation pigs spent more time active, and CE pigs tended to spend the most time inactive. Control gestation treatment pigs also displayed more active bouts and tended to display more standing and inactive bouts as well compared to ETOH pigs. This could be that CON pigs were more "restless," which has been assessed as fear (Fraser, 1974). Multiple assessments of the same behavior across different behavior tests (isolation, open field and mazes) suggests that CE pigs were more anxious than CC, EC and EE pigs. In experiment 1 maze II, increased activity led to increased completion of the maze, but in experiment 2 maze II these pigs were not quicker at the maze so their increased activity does suggest anxiety. In fact, more ETOH gestation treatment pigs completed the maze more than CON pigs, and there was a tendency for

more EE pigs to complete the maze, compared to CC and CE and tended to more than EC pigs.

Learning deficits in FASD increase the number of errors made when learning a new task or word list. In experiment 1 maze I memory, control pigs spent more time on track to the reward compared to ETOH pigs, and in experiment 2 maze II, CON gestation treatment pigs also spent more time on track. The fact that CON pigs spent more time on track indicates fewer errors were made, but also could be because ETOH pigs were quicker to complete the maze and therefore spent overall less time in the maze, however this was not significant. As repetition increased, pigs, regardless of prenatal treatment, did make fewer wrong turns in experiment 1 maze I and maze II. In experiment 2, the pigs that were reared on ETOH sows made more errors but this only reached significance for maze I memory. Although it was not significant for the gestation by lactation treatment interactions, EE pigs made the most wrong turns. Prenatal exposure to ethanol increased the number of errors made in a maze, but it did not mean that the animal took longer to complete the maze (Reyes et al., 1989). The type of cued navigation could have affected the number of errors PAE offspring made in different maze types. Prenatally ethanol exposed rats made more errors in a radial arm maze when switching cued spatial conditions (Berman and Hannigan, 2000), and took longer when using distal cue spatial navigation, but there were no differences between control and PAE animals during proximal cued spatial navigation (Kelly et al., 1988; Cronise et al., 2001; Endres et al., 2005). Similar to this study, Sluyter et al.

(2005) and Reyes et al. (1989) found PAE rats to complete the maze quicker even though they made more errors.

Prenatal ethanol exposure caused hippocampal dysfunction which affects learning and memory. Abnormal hippocampal mossy fiber distribution (Fukui and Sakata-Haga, 2009) and decreased hippocampus capillary diameter (Kelly et al., 1990) have been found from fetal alcohol exposure. The amount of ethanol animals were prenatally exposed to may affect the degree of maze performance deficit. Reyes et al. (1989) found that all rats exposed to 17% ethanol reached the maze test criteria, but only 50% of the rats exposed to the higher 35% ethanol met criteria. Riley et al. (1979) also found a relationship between more replications needed in reverse learning in a T maze with increased ethanol consumption of the dam. The timing of ethanol exposure *in utero* may also affect spatial navigation and the hippocampus. Cronise et al. (2001) found only rats exposed the entire duration of gestation and postnatally in rat development to correspond to human third trimester, had longer latencies to reach the platform than controls or any rats only exposed during the beginning, middle or end of gestation. Hippocampal cell number decreased the most in animals exposed to ethanol the entire duration of gestation or at the end of gestation (Tran and Kelly, 2003; Livy et al., 2003), suggesting the end of gestation was the most important in hippocampal development, when our sows drank the least. Sows in this free-choice study may have not drank enough or during the important stages of fetal development to affect the hippocampus in order to observe deficits in PAE offspring learning as assessed by completion time and the number of pigs that successfully completed each

maze. Sluyter et al. (2005) found no deficits in hippocampal neuroanatomy in three different strains of PAE mice. As little as 5% ethanol produced a synaptic plasticity deficit in the hippocampal dentate gyrus in Sprague-Dawley rats (Sutherland et al., 1997), so it is possible that low levels of ethanol exposure were capable of causing hippocampal deficits. In experiment 2 maze II, even though EE pigs were quicker at the maze they also made the most mistakes, which is in support of others who found PAE offspring were quicker to completion regardless that they made more errors, and concluded it was from hippocampal dysfunction.

Drinking behavior

At adolescence, individuals with FASD had specific personality characteristics such as poor judgement, inability to understand consequences, impulsivity, and sensation/novelty seeking (Mattson et al., 1999; Davis et al., 2011; Thackray and Tifft, 2001). Adolescence is a stressful phase of life, and coping with environmental or social challenges may be more challenging. Increased aggression and problems with impulse control at an early age in FASD children were predictive of alcohol abuse in adolescence and early adulthood (Gunzerath et al., 2011). The anxiolytic effects of ethanol increased alcohol use due to the increased stress during adolescence (Varlinskaya and Spear, 2010). Not only does stress and certain behavioral characteristics predict alcohol abuse at adolescence, but prenatal ethanol exposure increased postnatal responsiveness of ethanol (Foltran et al., 2011; Nizhnikov et al., 2006; Pueta et al., 2011). At the equivalence of adolescence, pigs in experiment 2

were given access to 20% (v/v) ethanol in both group and individual housing conditions. When pigs were in grouped pens, no differences in ethanol consumption were found, but when they had access to ethanol individually, EE pigs consumed more ethanol than CE and EC pigs but not CC. The mechanism of ethanol preference in PAE offspring is unknown, but associative learning may have a role, with the first taste during adolescence reinforcing the effects of ethanol (Pautassi et al., 2012).

Social housing conditions affect ethanol consumption. Long-term isolation increased consumption and socially deprived rats showed preference towards higher concentrations of ethanol (20%) (Wolffgramm and Heyne, 1991). This explains why in the present study, ethanol consumption only increased when pigs were individually housed. Isolation in social animals led to distress, so it is uncertain if drinking increased due to the psychotropic effects of ethanol or stress (Wolffgramm, 1990). However, drinking ethanol increased social investigation, chronic ethanol exposure in adolescence increased social investigation even at low doses and showed less social avoidance (Varlinskay and Spear, 2010).

Social status also influenced drug abuse behavior. Non-dominant rats consumed more than twice as much of ethanol than dominant rats (Wolffgramm and Heyne, 1991). This is in support of these data because EE pigs were submissive at the age drinking behavior was assessed, and they consumed the most amount of ethanol. Some investigators suggest socially submissive animals ingested more ethanol because of the stressors of social competition (Blanchard et al., 1987; Kudryavtseva et al., 1991); however social stress was not present in this study directly before consumption.

Conclusion

The behavioral responses of offspring prenatally exposed to ethanol in a pig model were described in the summary table (Table 33). Behavioral differences in anxiety, locomotion, social status and learning between control pigs and pigs prenatally exposed to ethanol were found. In some behavior tests there were differences between experiment 1 (non-cross-fostered pigs) and experiment 2 (cross fostered), suggesting rearing environment or the stress of cross fostering could influence behavioral responses. Pigs prenatally exposed to ethanol were less fearful or anxious, were more active, and were quicker to complete a series of mazes and completed more mazes in the allotted time, even if more errors were made as found in experiment 2. At an early age, ETOH pigs were aggressive and dominant, but at adolescence they became submissive and drank more ethanol when housed individually. This was the first research of its kind using a domestic pig model. These data were complementary and different from those when using a rodent model. Preliminary basic studies are needed to develop a good FASD drinking paradigm, and more pigs are needed for behavioral studies of FASD consequences. But this novel model has the potential to be a useful model for FASD.

Table 1. Leukocyte counts and percentages of control (CON) sows or sows given ethanol (ETOH) throughout gestation.

	Treatment			Period of Gestation					P-values		
Measure	**CON**	**ETOH**	**Pooled SEM**	**0**	**30**	**60**	**90**	**Pooled SEM**	**TRT**	**PERIOD**	**TRT* PERIOD**
Number of sows	8	8	-	16	16	16	12	-	-	-	-
WBC	1122	1091	70.0	$1325^{b, xy}$	$1115^{ab, y}$	$1119^{ab, y}$	$867^{a, x}$	94.2	0.76	0.024	0.98
Neutrophil, #	468	428	25.7	678^{b}	421^{b}	427^{b}	266^{a}	75.5	0.60	0.006	0.93
Lymphocyte, #	527	530	25.2	480	572	574	488	42.9	0.94	0.27	0.66
Monocyte, #	83.0	79.6	6.61	91.0	80.7	77.0	76.6	9.19	0.72	0.66	0.83
Eosinophil, #	39.3	54.1	10.00	$76.3^{b,x}$	$40.1^{ab,y}$	$41.0^{ab,xy}$	$29.4^{a,x}$	14.13	0.79	0.011	0.39
Basophil, #	2.78	2.20	0.581	2.69	3.84	1.94	1.48	0.716	0.92	0.46	0.90
Neutrophil, %	374	330	41.0	511^{b}	324^{a}	336^{a}	237^{a}	56.3	0.46	0.012	0.96
Lymphocyte, %	512	540	38.2	353^{a}	562^{ab}	556^{b}	630^{b}	51.0	0.61	0.003	0.89
N:L	2.54	1.07	0.909	3.17^{a}	2.72^{b}	0.74^{b}	0.59^{b}	1.353	0.56	0.013	0.97
Monocyte, %	77.3	79.0	7.27	67.5^{a}	75.3^{a}	71.2^{a}	98.5^{b}	8.26	0.88	0.045	0.82
Eosinophil, %	37.3	51.3	8.62	65.7	38.6	36.5	36.3	13.06	0.62	0.41	0.43
Basophil, %	2.48	2.74	0.975	2.13	4.59	1.84	1.86	1.198	0.91	0.61	0.82

WBC = total white blood cell count.

N:L = neutrophil to lymphocyte count.

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts at the beginning of the alphabet are significantly different ($P < 0.05$) and at the end of the alphabet tend to be different ($P < 0.10$).

Table 2. Leukocyte counts and percentages of offspring from control (CON) and ethanol (ETOH) sows before and after weaning at 21 days of age.

	Treatment			Time			P-values		
Measure	**CON**	**ETOH**	**Pooled SEM**	**0**	**24**	**Pooled SEM**	**TRT**	**TIME**	**TRT* TIME**
Number of litters	4	4	-	8	8	-	-	-	-
WBC	972	1003	114.8	1009	966	89.6	0.85	0.59	0.79
Neutrophil, #	423	459	37.4	385^{a}	498^{b}	35.6	0.52	0.055	0.80
Lymphocyte, #	488	482	69.4	555^{b}	415^{a}	53.9	0.89	0.005	0.39
Monocyte, #	58.4	59.0	10.61	64.7^{b}	52.7^{a}	8.34	0.87	0.041	0.64
Eosinophil, #	5.38^{y}	2.83^{x}	1.129	6.08^{b}	2.13^{a}	0.929	*0.08*	0.0004	0.99
Basophil, #	3.15	3.68	1.887	4.79	2.04	1.625	0.85	0.19	0.71
Neutrophil, %	442	442	15.3	387^{a}	498^{b}	15.3	0.99	0.002	0.59
Lymphocyte, %	494	495	11.4	554^{b}	445^{a}	13.0	0.95	0.003	0.66
N:L	0.96	0.98	0.060	0.74^{a}	1.20^{b}	0.062	0.78	0.002	0.90
Monocyte, %	58.4	59.6	3.77	63.3^{y}	54.8^{x}	3.40	0.84	*0.09*	0.69
Eosinophil, %	5.44^{b}	2.82^{a}	0.678	6.02^{b}	2.25^{a}	0.579	0.034	0.001	*0.09*
Basophil, %	3.99	4.22	2.157	6.05	2.15	1.904	0.98	0.47	0.45

WBC = total white blood cell counts.
N:L = neutrophil to lymphocyte counts.
Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts at the beginning of the alphabet are significantly different ($P < 0.05$) and at the end of the alphabet tend to be different ($P < 0.10$).

Table 3. Percent stress vocalizations of control (CON) and ethanol (ETOH), gilt and boar offspring being processed.

	Treatment			Sex			P-values		
Processing Procedure, % stress vocalizations	**CON**	**ETOH**	**Pooled SEM**	**GILT**	**BOAR**	**Pooled SEM**	**TRT**	**SEX**	**TRT* SEX**
Number of litters	8	8	-	-	-	-	-	-	-
Handle	11.9	11.0	2.60	10.5	12.4	2.18	0.75	0.53	0.45
Teeth	53.0	48.7	4.66	45.3[a]	56.4[b]	3.67	0.52	0.001	0.55
Tail	64.7	71.5	4.81	63.7[a]	72.5[b]	3.68	0.34	0.002	0.60
Identification	75.3	76.8	3.45	74.7	77.4	2.76	0.77	0.30	0.81
Injections	72.7	69.2	4.12	69.7	72.2	3.14	0.57	0.28	0.90
Castration	36.6	35.0	2.19	-	71.1	1.85	0.61	-	-
Total process	56.2	56.5	3.20	52.0[a]	60.8[b]	2.46	0.95	≤0.0001	0.97

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts differ ($P < 0.05$)

Table 4. Vocal response category of control (CON) and ethanol (ETOH) offspring in the backtest experiment 1.

Treatment	Number of litters	Low	Intermediate	High	P-value	Chi square	DF
CON	8	34	48	111	0.011	9.02	2
ETOH	8	49	30	78	-	-	-

Table 5. Escape response category of control (CON) and ethanol (ETOH) offspring in the backtest experiment 1.

Treatment	Number of litters	Low	Intermediate	High	P-value	Chi square	DF
CON	8	22	52	141	0.0001	18.07	2
ETOH	8	42	22	111	-	-	-

Table 6. Duration (sec) of control (CON) and ethanol (ETOH) offspring behaviors in a 2 hour isolation test experiment 1.

Duration of Behaviors, sec	**CON**	**ETOH**	**Pooled SEM**	**P-value**
Number of litters	6	6	-	-
Stand	2835	2152	315.3	0.32
Inactive	5527	5799	395.8	0.63
Active	1366[b]	857[a]	171.1	0.019
Vocal	137	110	46.60	0.81
Escape	394	402	105.7	0.24
Latency to "relax"	2224	2172	464.9	0.61

Data are expressed as LSMeans ± pooled SEM. Means without common subscripts differ ($P < 0.05$)

Table 7. Duration (sec) of control (CON) and ethanol (ETOH) offspring in the familiarization and novel periods of the open field test experiment 1.

	Treatment			Period			P-values		
Measure, sec	**CON**	**ETOH**	**SEM**	**Familiar**	**Novel**	**SEM**	**TRT**	**Period**	**TRT* Period**
Number of litters	6	6	-	12	12	-	-	-	-
Square 1	33.5	24.6	6.69	37.4^{b}	20.8^{a}	5.66	0.37	0.010	*0.09*
Square 2	23.3	21.0	5.56	25.2	19.1	4.889	0.12	0.72	0.67
Square 3	28.8	32.1	5.47	35.6	25.4	5.21	0.68	0.15	0.75
Square 4	29.0	25.0	3.13	32.9^{b}	21.1^{a}	3.67	0.40	0.049	0.49
Square 5	31.9	25.0	6.78	17.7	39.3	6.66	0.38	0.17	0.46
Square 6	52.9	47.2	7.16	30.8^{a}	69.3^{b}	6.81	0.59	<0.0001	0.28
Square 7	55.9	59.3	15.88	72.4^{b}	42.8^{a}	13.58	0.93	0.004	0.26
Square 8	38.0	34.4	7.56	39.7	32.7	6.88	0.74	0.42	1.00
Square 9	63.0	45.1	15.16	59.8	48.2	13.54	0.38	0.20	0.57
Stand	219^{a}	318^{b}	24.3	263	274	24.4	0.028	0.75	0.30
Inactive	293	261	51.9	281	273	38.9	0.68	0.759	0.68
Active	89.1^{x}	130.5^{y}	13.4	126^{b}	93.6^{a}	11.97	0.06	0.032	0.49
Escape attempt	0.45	0.49	0.350	0.42	0.53	0.273	0.91	0.80	0.13

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts within a section differ ($P < 0.05$). Square 5 is the middle of the arena, Square 6 where the novel object is placed, and Square 7 the "entrance" to the test.

Table 8. Frequency (No.) of control (CON) and ethanol (ETOH) offspring behaviors in the familiarization and novel periods of the open field test experiment 1.

	Treatment			Period			P-values		
Measure, #	**CON**	**ETOH**	**Pooled SEM**	**Familiar**	**Novel**	**SEM**	**TRT**	**Period**	**TRT* Period**
Number of litters	6	6	-	12	12	-	-	-	-
Square 1	3.31	2.83	0.671	4.39[b]	1.75[a]	0.528	0.62	<0.0001	0.45
Square 2	3.56	2.90	0.488	4.52[b]	1.94[a]	0.428	0.36	<0.0001	0.21
Square 3	3.15	2.72	0.453	4.10[b]	1.77[a]	0.389	0.52	<0.0001	0.64
Square 4	4.57	4.22	0.849	5.98[b]	2.81[a]	0.688	0.78	<0.0001	0.79
Square 5	3.14	3.33	0.493	3.46	3.01	0.408	0.78	0.30	0.45
Square 6	4.98	4.41	0.711	5.33[b]	4.06[a]	0.588	0.58	0.043	0.60
Square 7	4.46	4.31	0.547	6.10[b]	2.66[a]	0.500	0.86	<0.0001	0.61
Square 8	4.53	4.54	0.616	6.14[b]	2.93[a]	0.567	0.99	<0.0001	0.78
Square 9	4.02	3.26	0.485	4.63[b]	2.66[a]	0.418	0.30	0.0001	0.76
Stand	12.6	11.0	1.15	12.9[y]	10.6[x]	1.06	0.34	0.09	0.40
Inactive	12.6	11.1	1.18	13.1[y]	10.6[x]	1.08	0.39	0.08	0.37
Active	11.5	9.83	1.128	11.2	10.1	1.07	0.34	0.46	0.51
Escape	0.14	0.02	0.050	0.04	0.13	0.056	0.14	0.32	*0.10*

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts within a section differ ($P < 0.05$). Square 5 is the middle of the arena, Square 6 where the novel object is placed, and Square 7 the "entrance" to the test.

Table 9. Social rank of control (CON) and ethanol (ETOH) offspring in a food competition test in experiment 1.

	Social Rank							
Treatment	Number of litters	1*	2	3	4	P-Value	Chi Square	DF
CON	8	13	8	7	1	0.002	14.96	3
ETOH	8	3	9	5	11	-	-	-

Table 10. Duration (sec) of behaviors for control (CON) and ethanol (ETOH) offspring across repetitions in Maze I experiment 1.

	Treatment			Maze Repetition					P-values		
Measure, sec	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**3**	**4**	**Pooled SEM**	**TRT**	**REP**	**TRT *REP**
Number of litters	8	8	-	15	15	15	15	-	-	-	-
Active	642	644	87.4	575	708	638	650	73.1	0.98	0.33	0.51
Stand	228	202	36.8	224	201	227	209	25.5	0.30	0.71	0.039
Inactive	250	220	33.9	233	225	242	241	31.5	0.53	0.96	0.30
On track	590	583	84.1	499	622	617	608	72.4	0.95	0.22	0.79
Wrong turn	241	250	30.8	266	217	246	254	31.1	0.85	0.69	0.61
Near reward	33.7	14.3	10.13	22.7	34.8	10.5	27.9	10.23	0.20	0.23	0.23
Total duration	460	441	36.9	455	447	445	456	38.1	0.95	0.83	0.58
Latency to near reward	291	276	61.4	289	217	313	317	53.2	0.84	0.13	0.54

Data are expressed as LSMeans ± pooled SEM.

Table 11. Duration (sec) of behaviors for control (CON) and ethanol (ETOH) offspring across repetitions in Maze I Memory experiment 1.

	Treatment			Maze Repetition			P-values		
Measure, sec	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**Pooled SEM**	**TRT**	**REP**	**TRT* REP**
Number of litters	8	8	-	15	15	-	-	-	-
Active	544	528	96.8	542	530	75.2	0.91	0.85	*0.09*
Stand	173	144	38.5	159	159	29.2	0.60	1.00	0.26
Inactive	211	170	36.6	181	200	29.0	0.43	0.50	0.16
On track	535	473	92.4	502	506	71.1	0.64	0.95	0.018
Wrong turn	207	192	36.3	198	201	30.2	0.76	0.92	0.62
Near reward	13.0	20.0	5.38	11.4	21.6	5.43	0.38	0.21	0.54
Total duration	380	348	54.8	360	368	44.4	0.62	0.91	0.22
Latency near reward	290	140	45.4	221	209	37.7	0.13	0.87	0.89

Data are expressed as LSMeans ± pooled SEM.

Table 12. Duration (sec) of behaviors for control (CON) and ethanol (ETOH) offspring across repetitions in Maze II experiment 1.

	Treatment			Maze Rep					P-values		
Measure, sec	CON	ETOH	Pooled SEM	1	2	3	4	Pooled SEM	TRT	REP	TRT* REP
Number of litters	8	8	-	15	15	15	15	-	-	-	-
Active	572	523	97.6	$643^{b,xy}$	$595^{ab,y}$	$488^{a,xy}$	$464^{a,x}$	81.7	0.73	0.044	0.85
Stand	116	99.3	25.0	130	113	100	87.7	21.0	0.65	0.12	0.30
Inactive	161	132	36.0	152	175	122	137	29.5	0.58	0.23	0.50
On track	577	498	106.4	$614^{c,xy}$	$608^{bc,y}$	$472^{ab,y}$	$457^{a,xy}$	86.8	0.61	0.051	0.58
Wrong turn	131	126	30.5	154	143	110	108	27.0	0.92	0.20	0.89
Near reward	10.4	17.3	4.63	16.9	5.96	14.0	18.5	5.234	0.31	0.33	0.87
Total duration	349	314	65.8	384^{b}	371^{b}	290^{a}	281^{a}	53.5.	0.80	0.017	0.77
Latency near reward	289	216	51.7	273^{b}	336^{b}	188^{a}	214^{a}	46.40	0.50	0.007	0.87

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts at the beginning of the alphabet significantly differ ($P < 0.05$) and at the end of the alphabet tend to differ ($P < 0.10$).

Table 13. Frequency (No.) of behaviors for control (CON) and ethanol (ETOH) offspring across repetitions in Maze I experiment 1.

	Treatment			Maze Repetition					P-values		
Measure, #	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**3**	**4**	**Pooled SEM**	**TRT**	**REP**	**TRT* REP**
Number of litters	8	8	-	15	15	15	15	-	-	-	-
Active	11.0	10.6	2.96	$12.4^{b,y}$	$9.98^{ab,x}$	$11.1^{ab,xy}$	$9.65^{a,xy}$	2.348	0.95	*0.06*	0.73
Stand	9.83	9.37	3.083	11.2^{b}	8.68^{a}	9.93^{ab}	8.55^{a}	2.550	0.98	0.043	0.74
Inactive	10.0	9.44	3.082	11.4^{b}	8.80^{a}	10.1^{ab}	8.72^{a}	2.552	0.97	0.06	0.72
Wrong turn	3.75	3.41	0.755	$4.54^{b,y}$	$3.40^{ab,x}$	$3.39^{a,xy}$	$2.99^{a,xy}$	0.63	0.75	0.044	0.89
Near reward	0.85	0.82	0.166	1.06	0.91	0.67	0.71	0.174	0.47	0.38	0.053
Completed maze	0.39	0.40	0.096	0.42	0.37	0.35	0.44	0.090	0.91	0.73	0.68

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts at the beginning of the alphabet significantly differ ($P < 0.05$) and at the end of the alphabet tend to differ ($P < 0.10$).

Table 14. Frequency (No.) of behaviors for control (CON) and ethanol (ETOH) offspring across repetitions in Maze I Memory experiment 1.

	Treatment			Maze Rep			P-values		
Measure, #	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**Pooled SEM**	**TRT**	**REP**	**TRT* REP**
Number of litters	8	8	-	15	15	-	-	-	-
Active	9.01	7.74	2.291	8.48	8.26	1.875	0.52	0.54	0.64
Stand	7.85	6.41	2.450	7.24	7.02	1.986	0.47	0.46	0.83
Inactive	8.26	6.59	2.461	7.57	7.28	2.004	0.39	0.46	0.82
Wrong turn	2.64	2.60	0.604	2.81	2.43	0.490	0.96	0.44	0.94
Near reward	0.82	1.01	0.155	0.83	1.01	0.137	0.92	0.45	0.50
Completed maze	0.51	0.56	0.091	0.51	0.57	0.079	0.72	0.63	0.038

Data are expressed as LSMeans ± pooled SEM.

Table 15. Frequency (No.) of behaviors for control (CON) and ethanol (ETOH) offspring across repetitions in Maze II experiment 1.

	Treatment			Maze Repetition					P-values		
Measure, #	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**3**	**4**	**Pooled SEM**	**TRT**	**REP**	**TRT *REP**
Number of litters	8	8	-	15	15	15	15	-	-	-	-
Active	5.95	5.61	0.724	6.58	5.72	5.57	5.23	0.619	0.67	0.13	0.97
Stand	4.63	4.17	0.717	5.20	4.29	4.19	3.91	0.609	0.60	0.13	0.89
Inactive	4.99	4.26	0.777	5.44	4.40	4.41	4.25	0.656	0.52	0.13	0.86
Wrong turn	1.80	1.69	0.413	$2.36^{b,y}$	$1.66^{ab,x}$	$1.65^{a,xy}$	$1.32^{a,xy}$	0.365	0.85	0.029	0.82
Near reward	0.69	0.88	0.137	0.78	0.53	0.93	0.91	0.123	0.97	0.35	0.75
Completed maze	0.58	0.69	0.133	0.51	0.66	0.66	0.68	0.107	0.57	0.11	0.98

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts at the beginning of the alphabet significantly differ ($P < 0.05$) and at the end of the alphabet tend to differ ($P < 0.10$).

Table 16. Backtest measures of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatments in experiment 2.

	Gestation Treatment			Lactation Treatment			P-values		
Measure	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**
Vocalizations, sec	18.1	15.7	4.47	16.5	17.2	3.79	0.72	0.86	0.18
Vocalizations, #	2.33	2.25	0.413	2.12	2.46	0.346	0.89	0.37	0.35
Escape attempts, sec	15.2	13.3	2.14	14.2	14.3	1.81	0.58	0.96	0.44
Escape attempts, #	2.73	3.05	0.452	2.98	2.80	0.382	0.63	0.68	0.96

Data are expressed as LSMeans ± pooled SEM.

Table 17. Vocal response category of offspring in the backtest experiment 2.

Treatment	Number of litters	Low	Intermediate	High	P-value	Chi Square	DF
CC	3	7	16	39	0.43	5.94	6
CE	3	2	4	15	-	-	-
EC	3	5	4	9	-	-	-
EE	3	8	10	45	-	-	-

Treatments: gestation-lactation treatments: control-control (CC), control-ethanol (CE), ethanol-control (EC), ethanol-ethanol (EE).

Table 18. Escape response category of offspring in the backtest experiment 2.

Treatment	Number of litters	Low	Intermediate	High	P-value	Chi Square	DF
CC	3	2	24	42	0.001	22.39	6
CE	3	2	4	15	-	-	-
EC	3	4	2	15	-	-	-
EE	3	5	6	60	-	-	-

Treatments: gestation-lactation treatments: control-control (CC), control-ethanol (CE), ethanol-control (EC), ethanol-ethanol (EE).

Table 19. Duration (sec) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatments in the 2 hour isolation test in experiment 2.

	Gestation Treatment			Lactation Treatment			P-values		
Duration, sec	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**
Stand	2953	1973	442.9	1890^{x}	3036^{y}	442.9	0.13	*0.08*	*0.08*
Inactive	5311	5605	653.9	4997	5920	653.9	0.75	0.33	0.26
Active	1631^{y}	903^{x}	271.7	1153	1381	271.7	*0.07*	0.56	0.76
Vocalize	148	111	76.1	102	157	72.3	0.73	0.24	0.31
Escape	311	441	170.0	526	226	170.0	0.52	0.33	0.18
Latency to “relax”	2893	2891	582.9	2289	3495	582.9	1.00	0.15	0.80

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts tend to differ ($P < 0.10$).

Table 20. Frequency (No.) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatments in the 2 hour isolation test in experiment 2.

	Gestation Treatment			**Lactation Treatment**			**P-values**		
Frequency, #	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**
Stand	96.6	69.5	14.36	78.7	87.4	12.90	0.25	0.59	*0.08*
Inactive	124	97.3	23.58	112	109	19.3	0.47	0.89	0.16
Active	96.9^{y}	51.2^{x}	13.02	61.5	86.6	12.34	*0.07*	0.14	*0.09*
Vocalize	16.8	7.71	11.46	8.97	15.6	6.35	0.25	0.64	0.58
Escape	50	30.3	9.93	40.5	39.8	9.93	0.17	0.96	0.14

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts tend to differ ($P < 0.10$).

Table 21. Duration (sec) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatments and the familiarization (FAM) and novel (NOV) periods of the open field test experiment 2.

	Gestation Treatment			Lactation Treatment			Period			P-values						
Duration, sec	**CON**	**ETOH**	**SEM**	**CON**	**ETOH**	**SEM**	**FAM**	**NOV**	**SEM**	**GEST**	**LACT**	**GEST* LACT**	**PERIOD**	**GEST* PERIOD**	**LACT* PERIOD**	**GEST* LACT* PERIOD**
Square 1	22.6	24.4	5.43	24.8	22.2	4.76	28.6^{y}	18.5^{x}	4.73	0.82	0.65	0.39	*0.08*	0.18	0.62	0.19
Square 2	17.4	15.6	5.15	12.4	20.6	4.45	20.5	12.5	4.42	0.82	0.12	0.26	0.13	0.11	0.66	0.59
Square 3	21.2	26.9	5.06	24.0	24.1	4.79	33.3^{b}	14.8^{a}	4.76	0.47	0.98	0.20	0.006	0.62	0.07	0.46
Square 4	28.6	21.4	3.66	29.8	20.2	5.10	33.5^{y}	16.6^{x}	5.14	0.35	0.28	0.19	*0.07*	0.31	0.55	*0.10*
Square 5	40.1	20.1	9.71	28.5	31.6	10.13	15.2	44.9	10.07	0.42	0.51	0.33	0.19	0.34	0.93	0.50
Square 6	58.4	41.7	11.12	41.7	58.4	10.08	30.3^{a}	69.8^{b}	10.02	0.35	0.20	0.91	0.004	0.30	0.54	0.12
Square 7	35.4^{x}	54.5^{y}	3.76	51.7	38.2	5.09	61.1^{b}	28.7^{a}	5.11	*0.08*	0.13	0.39	0.001	0.26	0.66	*0.07*
Square 8	49.9^{b}	23.6^{a}	4.54	39.5	34.0	6.02	40.2	33.4	6.04	0.044	0.59	0.39	0.51	0.65	0.81	0.33
Square 9	52.4	56.3	14.87	56.2	52.5	12.87	45.9	62.8	12.80	0.93	0.69	0.87	0.54	0.48	0.63	0.26
Stand	227	320	29.3	271	276	31.9	272	275	31.5	0.16	0.92	0.68	0.95	0.70	0.70	0.51
Inactive	233	321	31.6	272	281	32.2	271	283	31.8	0.16	0.85	0.54	0.80	0.62	0.82	0.55
Active	101	110	17.5	96.0	115	16.6	125	85.4^{x}	16.5	0.73	0.40	0.60	*0.08*	0.76	0.73	0.76

Data are expressed as LSMeans ± SEM. Means within a section without common subscripts at the beginning of the alphabet significantly differ ($P < 0.05$) and at the end of the alphabet tend to differ ($P < 0.10$). Square 5 is the middle of the arena, Square 6 where the novel object was placed, and Square 7 the "entrance" to the test.

Table 22. Frequency (No.) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatments in the familiarization (FAM) and novel (NOV) periods in the open field test experiment 2.

	Gestation Treatment			Lactation Treatment			Period			P-values						
Frequency, #	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**SEM**	**FAM**	**NOV**	**SEM**	**GEST**	**LACT**	**GEST* LACT**	**PERIOD**	**GEST* PERIOD**	**LACT* PERIOD**	**GEST* LACT* PERIOD**
Square 1	4.83	2.83	0.845	3.92	3.75	0.689	5.18^{b}	2.48^{a}	0.686	0.16	0.81	0.59	0.0004	0.66	0.19	0.11
Square 2	4.22	3.21	0.737	4.04	3.40	0.633	5.14^{b}	2.29^{a}	0.630	0.38	0.38	0.83	0.0004	0.15	0.24	0.44
Square 3	3.82	3.10	0.728	3.6	3.35	0.605	4.68^{b}	2.23^{a}	0.601	0.53	0.73	0.73	0.001	0.69	0.58	0.31
Square 4	6.39	4.48	1.175	5.04	5.83	1.00	6.94^{b}	3.94^{a}	0.990	0.31	0.48	0.52	0.010	0.93	0.93	0.36
Square 5	3.74	3.24	0.758	3.91	3.06	0.597	3.69	3.29	0.595	0.67	0.12	0.08	0.44	1.00	0.85	0.85
Square 6	6.06	5.01	0.867	5.81	5.26	0.747	6.13	4.93	0.742	0.45	0.52	0.77	0.16	0.41	0.41	0.35
Square 7	5.52	4.51	0.751	4.52	5.51	0.595	6.64^{b}	3.39^{a}	0.591	0.29	0.30	0.30	0.002	0.71	0.79	0.20
Square 8	5.56	4.69	0.254	4.44	5.82	0.532	6.70^{b}	3.55^{a}	0.548	0.22	0.18	0.14	0.005	0.47	0.81	0.60
Square 9	4.62	3.51	0.647	6.63	4.50	0.569	4.94^{b}	3.19^{a}	0.566	0.30	0.51	0.85	0.014	0.71	0.82	0.82
Stand	14.2	11.8	1.09	12.6	13.4	1.27	13.5	12.6	1.26	0.23	0.70	0.19	0.66	0.62	0.80	1.00
Inactive	14.2	12.0	1.18	12.7	13.5	1.32	13.6	12.6	1.31	0.27	0.69	0.20	0.61	0.58	0.79	0.98
Active	13.4^{b}	11.5^{a}	0.08	12.0	12.9	0.97	12.3	12.6	1.01	0.036	0.63	*0.10*	0.86	0.68	0.71	0.86

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts at the beginning of the alphabet significantly differ ($P < 0.05$) and at the end of the alphabet tend to differ ($P < 0.10$). Square 5 is the middle of the arena, Square 6 where the novel object was placed, and Square 7 the "entrance" to the test.

Table 23. Teat order of offspring in experiment 2.

Treatment	Number of litters	Cranial	Middle	Caudal	P-value	Chi Square	DF
CC	3	0	3	2	*0.093*	10.9	6
CE	3	0	3	2	-	-	-
EC	3	0	5	0	-	-	-
EE	3	2	3	0	-	-	-

Treatments: gestation – lactation treatments; control-control (CC), control-ethanol (CE), ethanol-control (EC), and ethanol-ethanol (EE).

Table 24. Dominance order of offspring in the socialization test in experiment 2.

Treatment	Number of litters	DOM	SUB	P-value	Chi Square	DF
CC	3	1	3	0.050	7.8	3
CE	3	1	4	-	-	-
EC	3	5	0	-	-	-
EE	3	2	2	-	-	-

DOM = dominant rank.
SUB = submissive rank.
Treatments: gestation – lactation treatments; control-control (CC), control-ethanol (CE), ethanol-control (EC), and ethanol-ethanol (EE).

Table 25. Social rank of offspring in the food competition test in experiment 2.

Treatment	Number of litters	DOM	INT 2	INT 3	SUB	P-value	Chi Square	DF
CC	3	3	1	0	1	0.03	18.5	9
CE	3	2	2	1	0	-	-	-
EC	3	0	1	3	0	-	-	-
EE	3	0	2	0	3	-	-	-

DOM = dominant rank.
INT 2 = intermediate 2nd in rank.
INT 3 = intermediate 3rd in rank.
SUB = submissive rank.
Treatments: gestation – lactation treatments; control-control (CC), control-ethanol (CE), ethanol-control (EC), and ethanol-ethanol (EE).

Table 26. Duration (sec) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatment across repetitions in Maze I experiment 2.

	Gestation Treatment			Lactation Treatment			Maze Repetition					P-values						
Measure, sec	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**3**	**4**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**	**REP**	**GEST* REP**	**LACT* REP**	**GEST* LACT* REP**
Number of litters	3	3	-	3	3	-	6	6	6	6	-	-	-	-	-	-	-	-
Active	850	831	57.2	824	857	48.2	897	871	815	780	60.5	0.82	0.56	0.40	0.41	0.26	0.80	0.47
Stand	298	285	32.6	283	300	26.8	297	265	327	278	32.9	0.80	0.59	0.47	0.43	0.18	0.78	0.75
Inactive	304	294	35.3	293	305	28.7	303	276	327	290	34.8	0.84	0.68	0.72	0.61	0.31	0.80	0.72
On track	811	787	64.2	776	823	54.7	823	824	809	741	69.5	0.81	0.49	0.71	0.74	0.57	0.98	0.42
Wrong turn	324	313	35.2	319	319	30.2	361	282	326	306	38.6	0.84	0.99	0.72	0.45	0.82	0.91	0.93
Near reward	14.4	12.0	0.693	12.1	14.4	3.765	7.9	30.8	1.78	12.4	6.681	0.94	0.85	0.73	0.73	0.35	0.25	0.93
Time to completion	554	515	38.3	511	558	32.1	571	521	536	510	40.2	0.42	0.32	0.81	0.57	0.24	0.87	0.40
Latency near reward	434	449	50.0	422	461	42.7	$514^{b,xy}$	$322^{a,x}$	$499^{b,xy}$	$431^{ab,y}$	54.4	0.64	0.81	0.70	0.025	0.79	0.98	0.52

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts at the beginning of the alphabet significantly differ ($P < 0.05$) and at the end of the alphabet tend to differ ($P < 0.10$).

Table 27. Frequency (No.) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatments across repetitions in Maze I experiment 2.

	Gestation Treatment			Lactation Treatment			Maze Repetition					P-values						
Measure, #	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**3**	**4**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**	**REP**	**GEST* REP**	**LACT* REP**	**GEST* LACT* REP**
Number of litters	3	3	-	3	3	-	6	6	6	6	-	-	-	-	-	-	-	-
Active	12.1	10.9	0.73	10.9	12.1	0.71	12.4	11.7	11.7	10.2	0.99	0.34	0.27	*0.06*	0.46	0.57	0.48	0.92
Stand	10.8	9.71	0.711	9.70	10.9	0.709	11.3	10.3	10.4	9.10	1.001	0.32	0.31	*0.07*	0.52	0.69	0.52	0.97
Inactive	10.8	9.87	0.780	9.73	11.0	0.747	11.3	10.4	10.4	9.28	1.032	0.42	0.27	*0.06*	0.57	0.72	0.53	0.94
Wrong turn	4.19	3.35	0.570	3.53	4.01	0.465	4.25	3.94	3.34	3.54	0.567	0.35	0.37	0.13	0.54	0.86	0.81	0.97
Completed	0.17	0.21	0.115	0.24	0.15	0.093	0.16	0.51	0.16	0.26	0.112	0.81	0.29	0.29	0.84	0.70	0.97	0.68

Data are expressed as LSMeans ± pooled SEM.

Table 28. Duration (sec) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatments across repetitions in Maze I Memory experiment 2.

	Gestation Treatment			Lactation Treatment			Maze Repetition			P-values						
Measure, sec	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**	**REP**	**GEST* REP**	**LACT* REP**	**GEST* LACT* REP**
Number of litters	3	3	-	3	3	-	6	6	-	-	-	-	-	-	-	-
Active	803	658	102.1	714	746	87.0	791	669	86.3	0.37	0.76	0.90	0.27	0.56	0.62	0.60
Stand	327^{y}	212^{x}	33.1	275	265	31.7	278	261	31.5	*0.07*	0.82	0.12	0.72	0.71	0.97	0.36
Inactive	340	231	40.8	290	281	35.1	293	278	34.8	0.13	0.83	0.24	0.73	0.30	0.61	0.39
On track	833	593	104.6	718	708	86.1	749	677	85.4	0.18	0.92	0.39	0.45	0.21	0.90	0.96
Wrong turn	314	259	36.5	285	288	36.9	313	259	36.7	0.35	0.96	0.74	0.36	0.95	0.61	0.58
Time to completion	566	431	58.1	495	501	51.8	516	481	51.4	0.13	0.67	0.49	0.57	0.51	0.81	0.94
Latency near reward	381	217	79.6	238^{x}	359^{y}	65.9	334	263	65.5	0.12	*0.06*	*0.06*	0.34	0.11	0.88	0.98

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts tend to differ ($P < 0.10$).

Table 29. Frequency (No.) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatment across repetitions in Maze I Memory experiment 2.

	Gestation Treatment			Lactation Treatment			Maze Repetition			P-values						
Measure, #	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**	**REP**	**GEST* REP**	**LACT* REP**	**GEST* LACT* REP**
Number of litters	3	3	-	3	3	-	6	6	-	-	-	-	-	-	-	-
Active	9.78	8.18	1.012	8.58	9.37	0.946	9.42	8.54	0.940	0.33	0.56	0.39	0.51	0.58	0.99	0.68
Stand	8.40	6.95	1.056	7.41	7.94	0.956	8.10	7.25	0.949	0.39	0.68	0.24	0.51	0.76	0.92	0.57
Inactive	8.52	7.08	1.124	7.50	8.10	0.989	8.29	7.31	0.982	0.42	0.64	0.23	0.44	0.64	0.96	0.59
Wrong turn	3.69	3.47	0.702	2.30^{x}	4.17^{y}	0.558	3.53	3.63	0.556	0.83	*0.08*	0.22	0.85	0.63	0.65	0.37
Completed	0.24	0.42	0.173	0.26	0.40	0.147	0.25	0.41	0.145	0.49	0.41	0.50	0.30	0.52	0.38	0.94

Data are expressed as LSMeans ± pooled SEM. Means within a section without common tend to differ ($P < 0.10$).

Table 30. Duration (sec) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatments across repetitions in Maze II experiment 2.

	Gestation Treatment			Lactation Treatment			Maze Repetition					P-values						
Measure, sec	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**3**	**4**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**	**REP**	**GEST* REP**	**LACT* REP**	**GEST* LACT* REP**
Number of litters	3	3	-	3	3	-	6	6	6	6	-	-	-	-	-	-	-	-
Active	950[b]	703[a]	50.3	841	813	54.2	842	875	798	792	52.5	0.026	0.75	0.68	0.90	0.92	0.53	0.68
Stand	196	149	23.1	171	174	21.0	204	160	178	147	27.5	0.22	0.94	0.36	0.40	0.72	0.82	1.00
Inactive	262	221	41.7	238	245	32.8	231	238	236	261	37.9	0.53	0.82	*0.08*	0.84	0.54	0.51	0.55
On track	966[x]	710[y]	80.9	821	855	70.3	847	836	826	843	89.4	*0.09*	0.70	0.31	1.00	0.94	0.60	0.46
Wrong turn	223	198	33.7	223	199	28.6	203	271	188	182	35.6	0.63	0.50	0.98	0.34	0.86	0.82	0.31
Near reward	15.6	12.6	7.69	20.2	8.01	6.59	19.6	0	15.6	22.8	8.29	0.79	0.18	0.83	0.24	0.36	0.83	0.48
Time to completion	581	454	48.6	526	509	41.9	531	527	504	507	53.0	0.12	0.59	0.26	0.89	0.95	0.76	0.82
Latency near reward	438	374	75.2	407	405	61.0	333	544	336	411	73.1	0.47	0.72	0.14	0.33	0.95	0.66	0.57

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts at the beginning of the alphabet significantly differ ($P < 0.05$) and at the end of the alphabet tend to differ ($P < 0.10$).

Table 31. Frequency (No.) of behaviors of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatments across repetitions of Maze II experiment 2.

	Gestation Treatment			Lactation Treatment			Maze Repetition					P-values						
Measure, #	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**1**	**2**	**3**	**4**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**	**REP**	**GEST* REP**	**LACT* REP**	**GEST* LACT* REP**
Number of litters	3	3	-	3	3	-	6	6	6	6	-	-	-	-	-	-	-	-
Active	8.58^{b}	6.93^{a}	0.421	7.95	7.56	0.451	8.35	7.83	7.74	7.09	0.651	0.051	0.59	0.87	0.56	0.57	0.72	0.28
Stand	7.23^{y}	5.57^{x}	0.508	6.41	6.39	0.492	7.16	6.23	6.35	5.85	0.674	*0.08*	0.98	0.75	0.50	0.68	0.92	0.39
Inactive	7.72^{y}	5.73^{y}	0.672	6.77	6.69	0.594	7.58	5.94	6.80	6.60	0.772	*0.10*	0.92	0.38	0.51	0.39	0.87	0.47
Wrong turn	3.01	2.23	0.594	2.58	2.68	0.471	3.05	2.43	2.69	2.29	0.552	0.40	0.79	0.38	0.54	0.71	0.74	0.85
Completed	0.11^{a}	0.39^{b}	0.091	0.21	0.29	0.084	0.21	0.31	0.25	0.25	0.111	0.030	0.44	*0.09*	0.95	0.94	0.11	0.65

Data are expressed as LSMeans ± pooled SEM. Means within a section without common subscripts at the beginning of the alphabet significantly differ ($P < 0.05$) and at the end of the alphabet tend to differ ($P < 0.10$).

Table 32. Ethanol consumption in group housing of control (CON) and ethanol (ETOH) offspring by gestation and lactation treatment in experiment 2.

	Gestation Treatment			Lactation Treatment			P-values		
Measure	**CON**	**ETOH**	**Pooled SEM**	**CON**	**ETOH**	**Pooled SEM**	**GEST**	**LACT**	**GEST* LACT**
Number of litters	3	3	-	3	3	-	-	-	-
Ethanol, g/kg	0.16	0.32	0.125	0.22	0.26	0.104	0.44	0.75	0.44
Drink bouts, #	3.76	5.46	1.757	3.33	5.89	1.707	0.54	0.29	0.55
Time drinking, sec	70.8	38.6	28.91	34.0	75.4	28.91	0.44	0.33	0.70

Data are expressed as LSMeans ± pooled SEM.

Table 33. Summarization of all significant gestation main effects

Study	Test	Measure	Description	P-value
Ethanol Pharmacokinetics	Individual BEC	BEC, g/dL	Individual sows differed from one another	0.035
Ethanol Pharmacokinetics	BEC time	BEC, g/dL	Up to 550 min elevated compared to 0 min	<0.0001
FASD Sows	Sow ethanol consumption	Ethanol Consumption, g/kg	Starting at 11 weeks of gestation ethanol consumption decreased	0.002
FASD Sows	Reproduction	Reproduction	ETOH sows weighed more the week before farrowing	0.040
FASD Piglets Physiology/ Immunology	Immune	Neutrophil OB %	ETOH pigs had greater baseline percent of neutrophils positve for OB	0.014
FASD Processing	Processing	% Stress Vocalizations	Male pigs displayed more stress vocalizations during processing compared to females	<0.0001
FASD Piglets Behavior	Backtest Exp 1	Coping Style	More CON pigs were HR and IR	0.011
FASD Piglets Behavior	Backtest Exp 2	Coping Style	More ETOH pigs were HR	0.0004
FASD Piglets Behavior	Isolation Exp 1	Activity	ETOH pigs spent less time active	0.019
FASD Piglets Behavior	Open field Exp 1	Standing	ETOH pigs spent more time standing	0.028
FASD Piglets Behavior	Open field Exp 2	Activity	ETOH pigs displayed fewer active bouts	0.036
FASD Piglets Behavior	Social Status Exp 1	Food Competition Test	ETOH pigs were more submissive	0.020
FASD Piglets Behavior	Social Status Exp 2	Teat Order	ETOH pigs held cranial teats	0.015
FASD Piglets Behavior	Social Status Exp 2	Social Interaction	ETOH pigs won more fights	0.027
FASD Piglets Behavior	Social Status Exp 2	Food Competition Test	ETOH pigs were more submissive	0.028
FASD Piglets Behavior	Learning and Cognition	Maze I Exp 1	ETOH pigs spent more time standing in rep 1 compared to rep 2	0.039
FASD Piglets Behavior	Learning and Cognition	Maze I Exp 1	ETOH pigs were near the reward more times in rep 3 compared to CON	0.053
FASD Piglets Behavior	Learning and Cognition	Maze I Memory Exp 1	ETOH pigs less time on track in rep 2	0.018
FASD Piglets Behavior	Learning and Cognition	Maze I Memory Exp 1	ETOH pigs completed the maze more in rep 2	0.039
FASD Piglets Behavior	Learning and Cognition	Maze II Exp 2	ETOH pigs spent less time active	0.026
FASD Piglets Behavior	Learning and Cognition	Maze II Exp 2	ETOH pigs displayed fewer active bouts	0.051
FASD Piglets Behavior	Learning and Cognition	Maze II Exp 2	ETOH pigs completed the maze more	0.030
FASD Drinking	Adolescence Drinking	Ethanol Consumption	EE pigs drank more ETOH when individually housed compared to CE and EC but not CC pigs	0.030

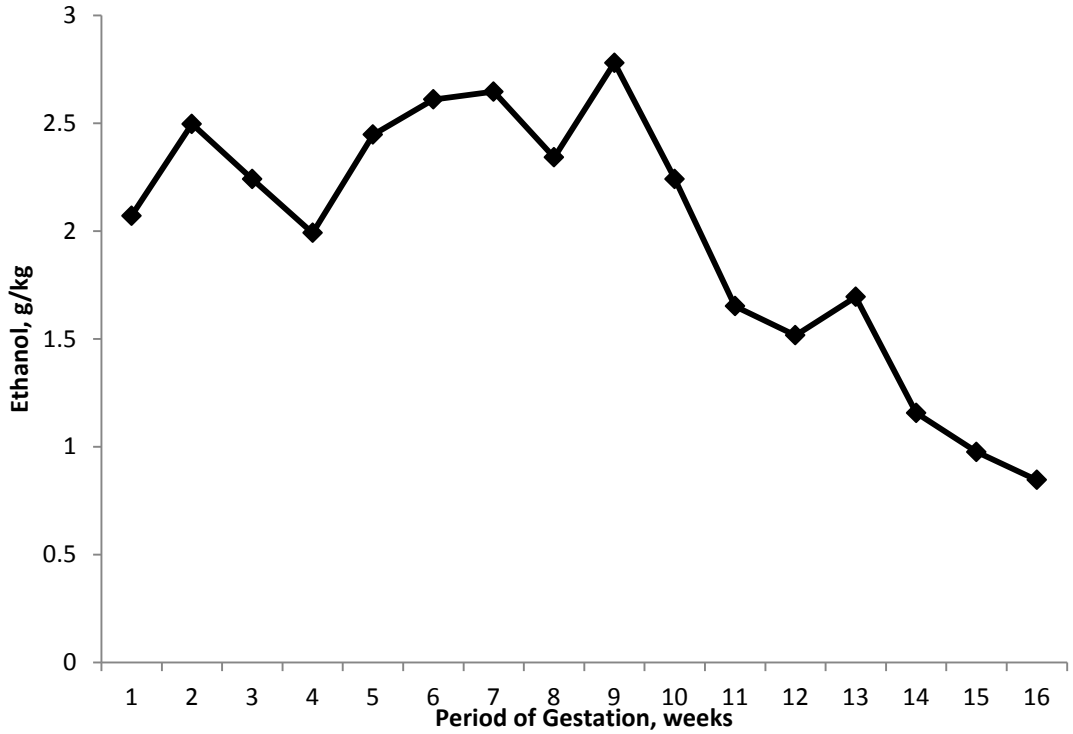

Figure 5. Average sow consumption throughout gestation. Data presented as LSMeans, SEM = 0.41.

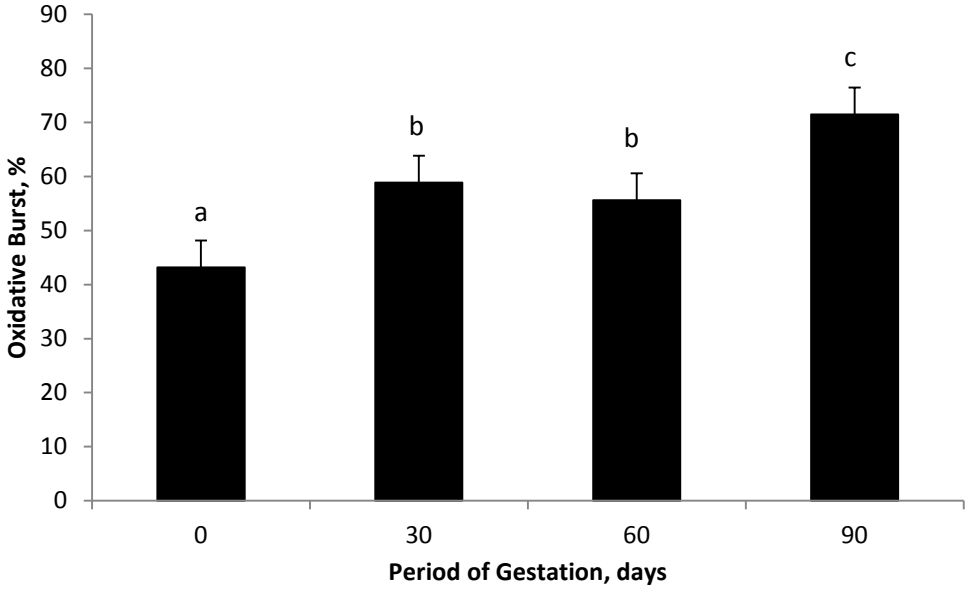

Figure 6. Percentage of neutrophils positive for oxidative burst in sows throughout gestation. Data are presented as LSMeans ± SEM. Means without common subscripts differ at $P < 0.05$.

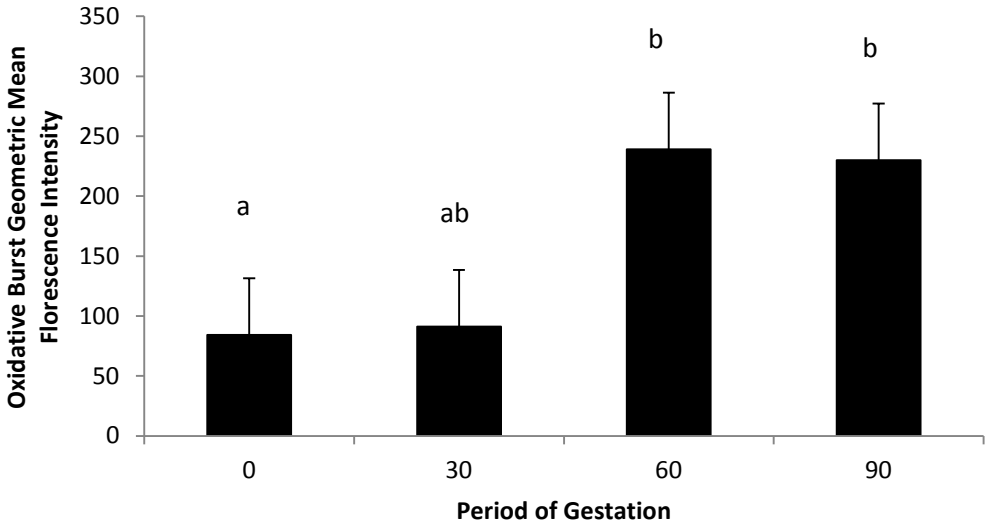

Figure 7. Geometric mean florescence intensity of neutrophils positive for oxidative burst. Data are presented as LSMeans ± SEM. Means without common subscripts differ at $P < 0.05$.

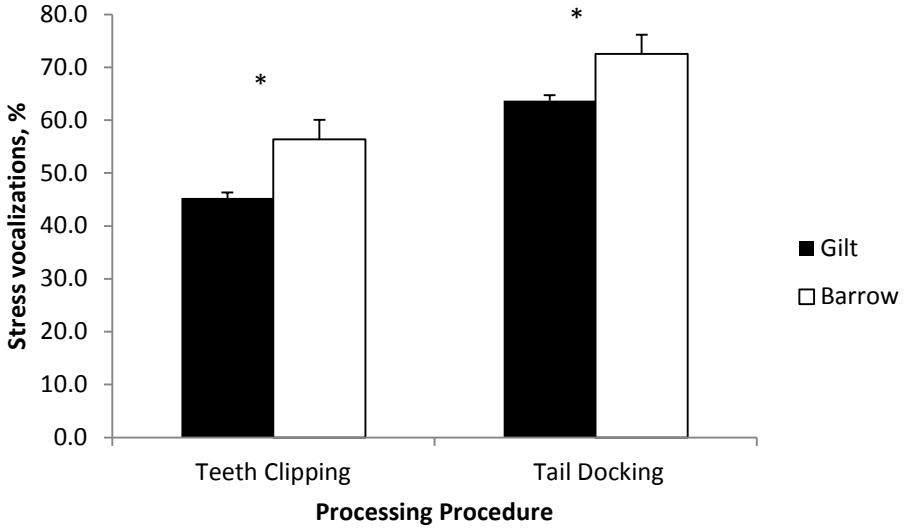

Figure 8. The percent stress vocalizations of gilts and barrows teeth clipped and tail docked during routine farm processing. Data are presented as LSMeans ± SEM. For LSMeans accompanied by an asterisk *signifies significant differences ($P < 0.05$) between gilts and boars.

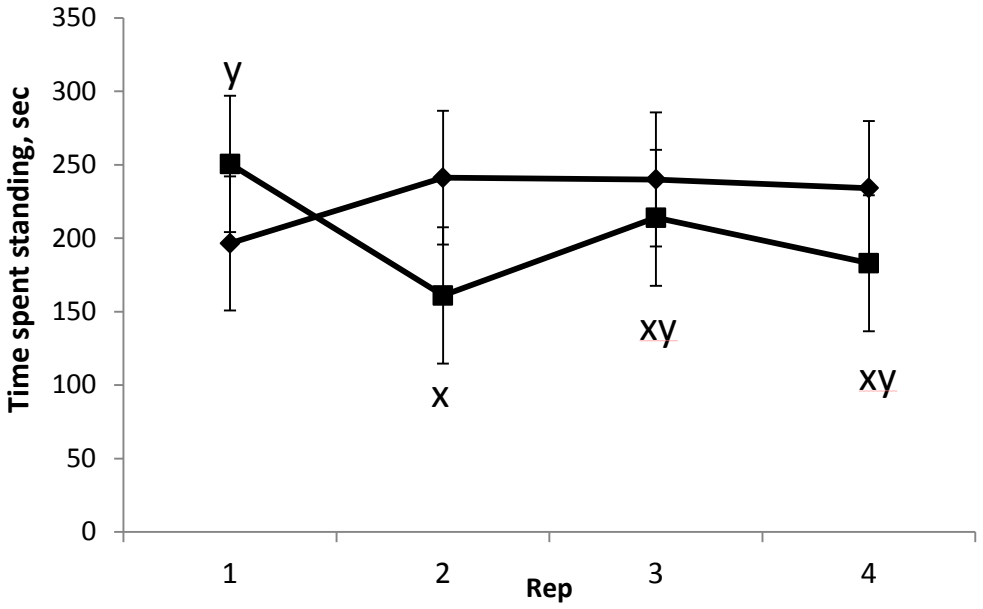

Figure 9. Treatment by repetition interaction for standing duration in Maze I experiment 1. Data presented as LSMeans ± SEM of control; diamonds ♦, and ethanol; squares ■, pigs. Means without common subscript tend to differ at $P < 0.10$ within a treatment.

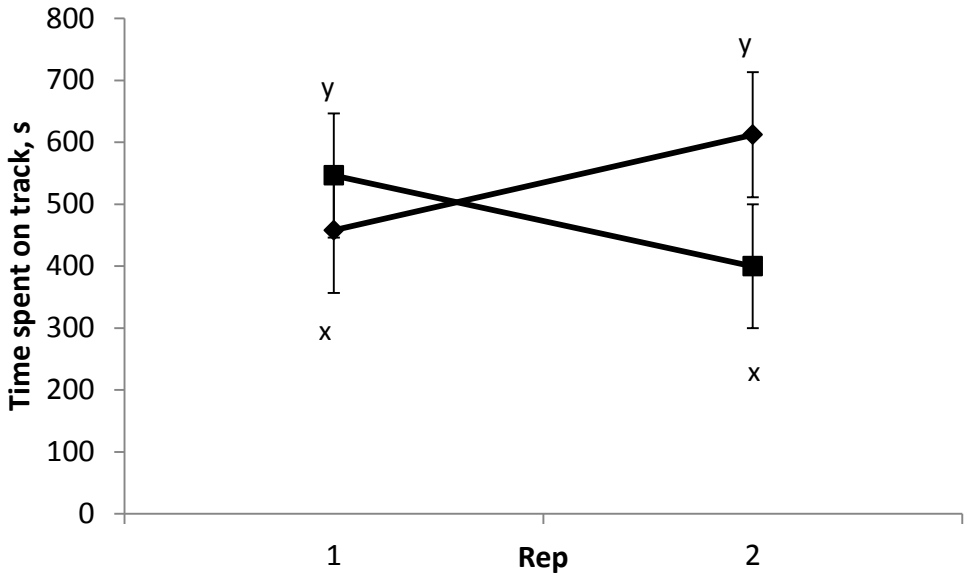

Figure 10. Treatment by repetition interaction for duration of time spent on track in Maze I Memory experiment 1. Data are presented as LSMeans ± SEM for control; diamonds ◆, and ethanol; squares ■ , pigs. Means without common subscripts tend to differ at $P < 0.10$ within a treatment.

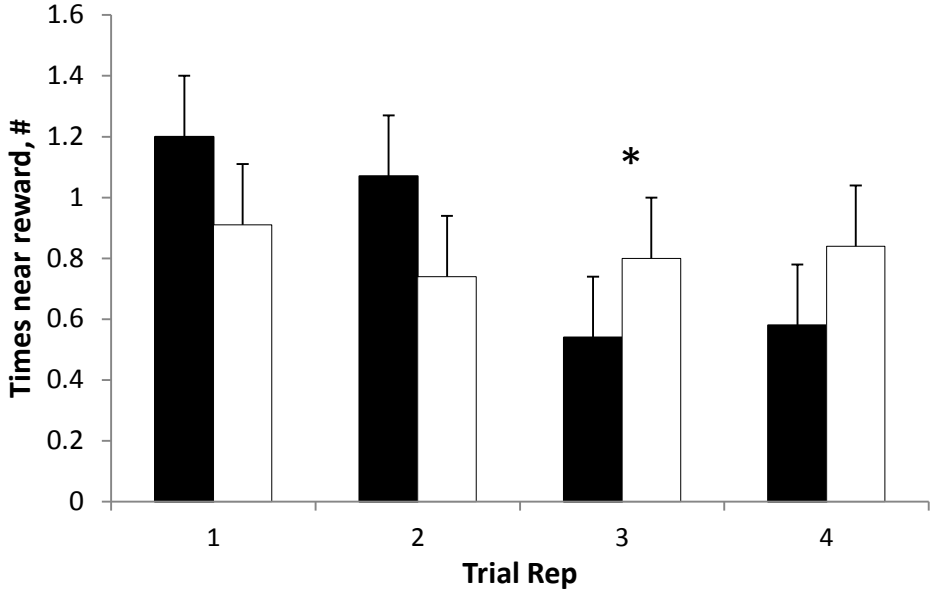

Figure 11. Treatment by repetition interaction for duration of time spent near the reward in Maze I experiment 1. Data are presented as LSMeans ± SEM for control; black bars, and ethanol, white bars. For LSMeans accompanied by an asterisk *signifies significant differences ($P < 0.05$) between control and ethanol pigs.

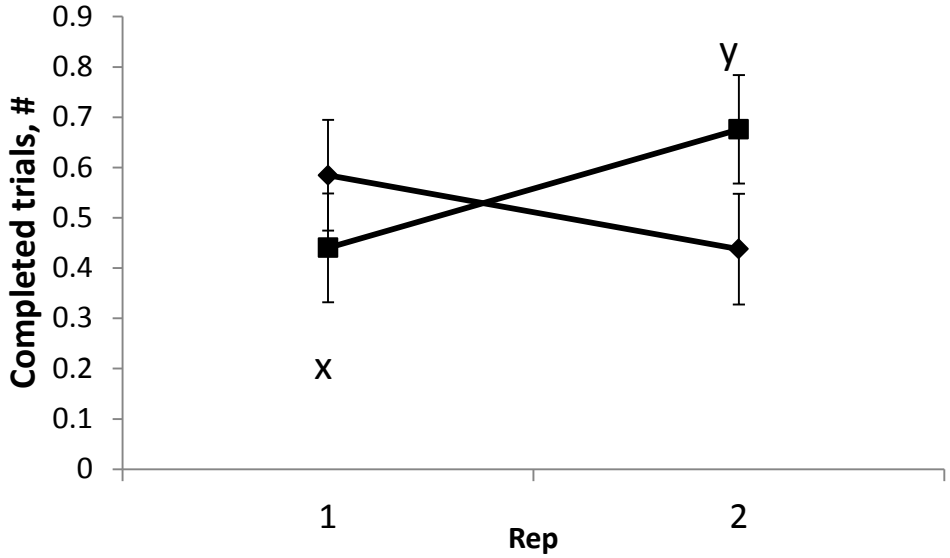

Figure 12. Treatment by repetition number of trials completed in Maze I Memory experiment 1. Data are presented as LSMeans ± SEM for control; diamonds ◆, and ethanol, squares ■, pigs. Means within a treatment without common subscripts tend to be different ($0.05 < P < 0.10$).

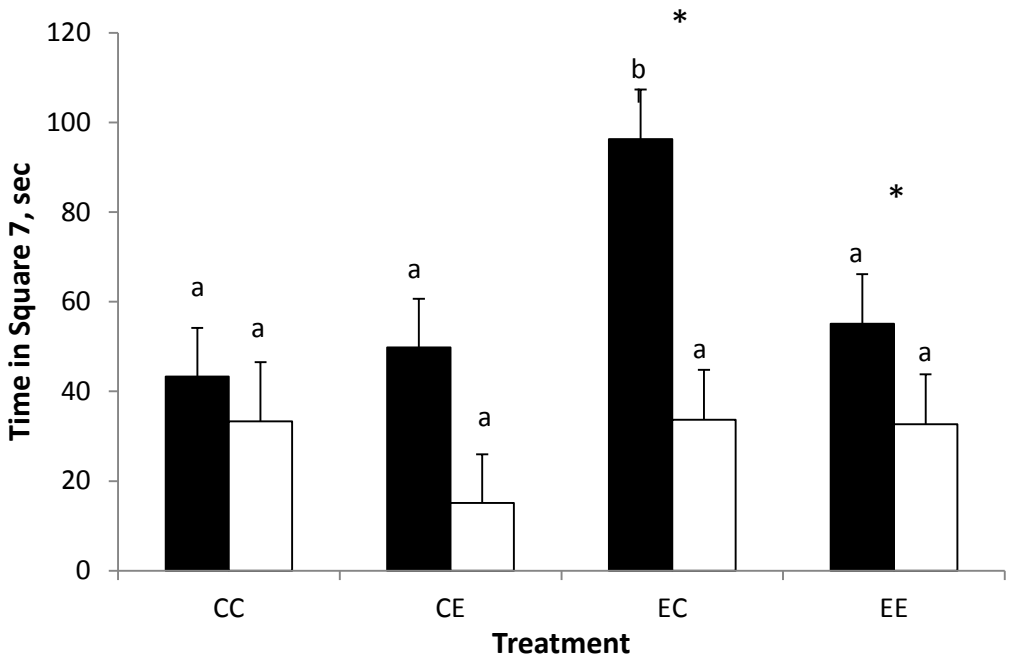

Figure 13. Gestation by lactation by period interaction of time spent standing in square 7, the entrance of the open field test in experiment 2. Data are presented as LSMeans ± SEM for control-control (CC), control-ethanol (CE), ethanolcControl (EC) and ethanol-ethanol (EE) pigs in the familiarization; black bars, and novel; white bars, periods. Means within a period without a common subscript differ at $P < 0.05$. Means accompanied by an asterisk * differ at $P < 0.05$ between periods within a treatment.

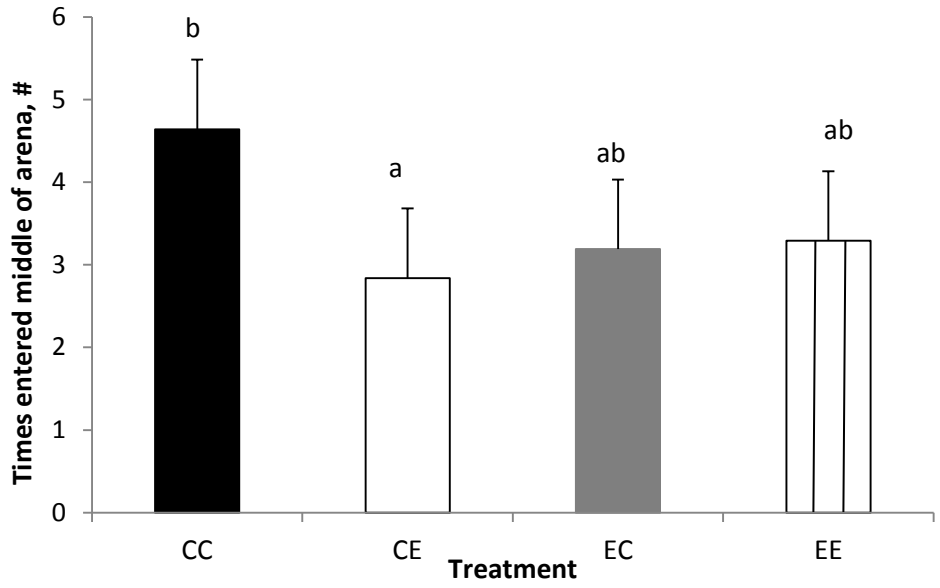

Figure 14. Gestation by lactation treatment interaction for the number of times entered the middle of the open field arena in experiment 2. Data are presented as LSMeans ± SEM for control-control (CC; black bars), control-ethanol (CE; white bars), ethanol-control (EC; grey bars), and ethanol-ethanol (EE; lined bars). Means without common subscripts differ at $P < 0.05$.

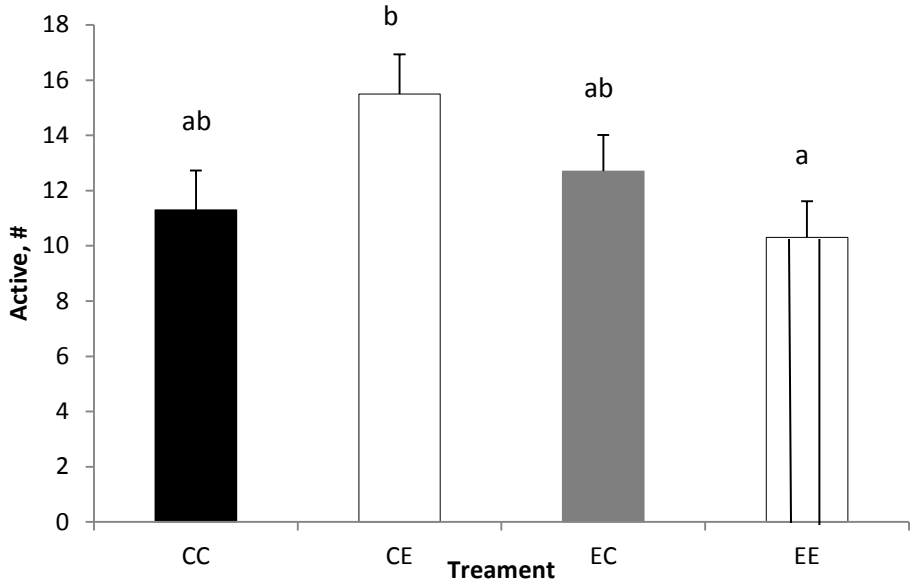

Figure 15. Gestation by lactation interaction for the number of active bouts in the open field test in experiment 2. Data are presented as LSMeans ± SEM for control-control (CC; black bars), control-ethanol (CE; white bars), ethanol-control (EC; grey bars), and ethanol-ethanol (EE; lined bars). Means without a common subscript differ at $P < 0.05$.

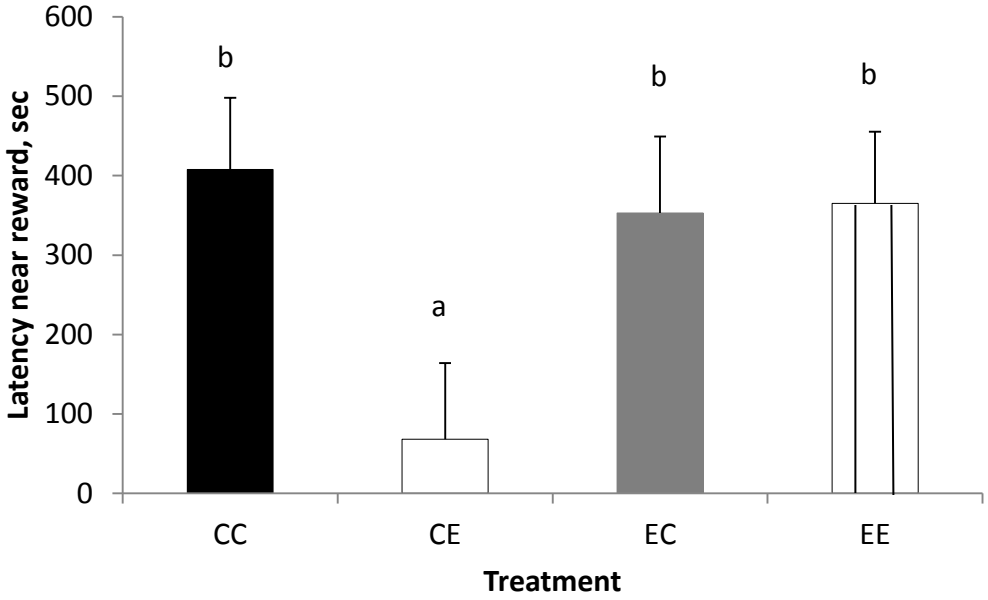

Figure 16. Latency to approach within 0.30 m of the reward for Maze I Memory experiment 2. Data are presented as LSMeans ± SEM for control-control (CC; black bars), control-ethanol (CE; white bars), ethanol-control (EC; grey bars) and ethanol-ethanol (EE; lined bars) pigs. Means without a common subscript differ at $P < 0.05$.

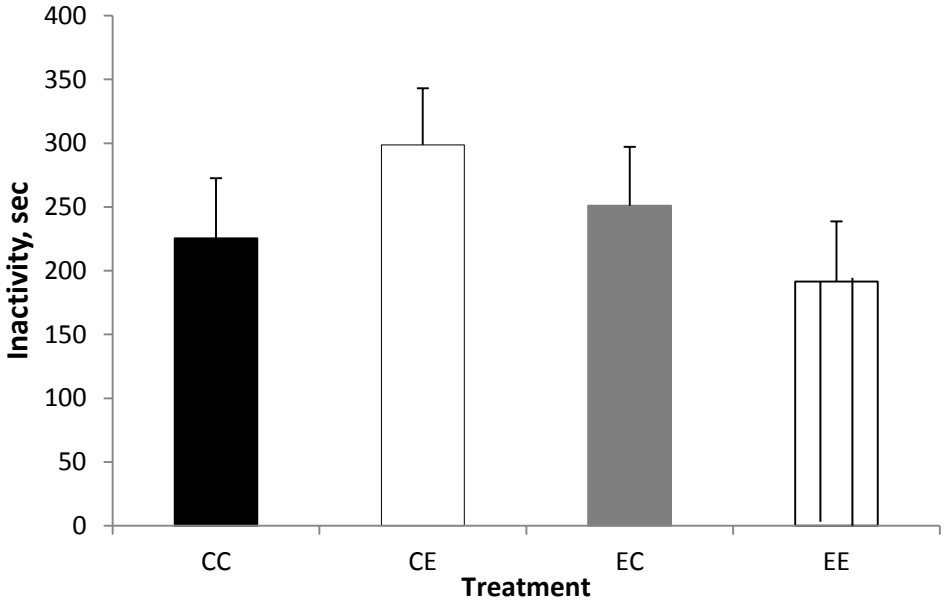

Figure 17. Time spent inactive in Maze II experiment 2. Data are presented as LSMeans ± SEM for control-control (CC; black bars), control-ethanol (CE; white bars), ethanol-control (EC; grey bars) and ethanol-ethanol (EE; lined bars) pigs.

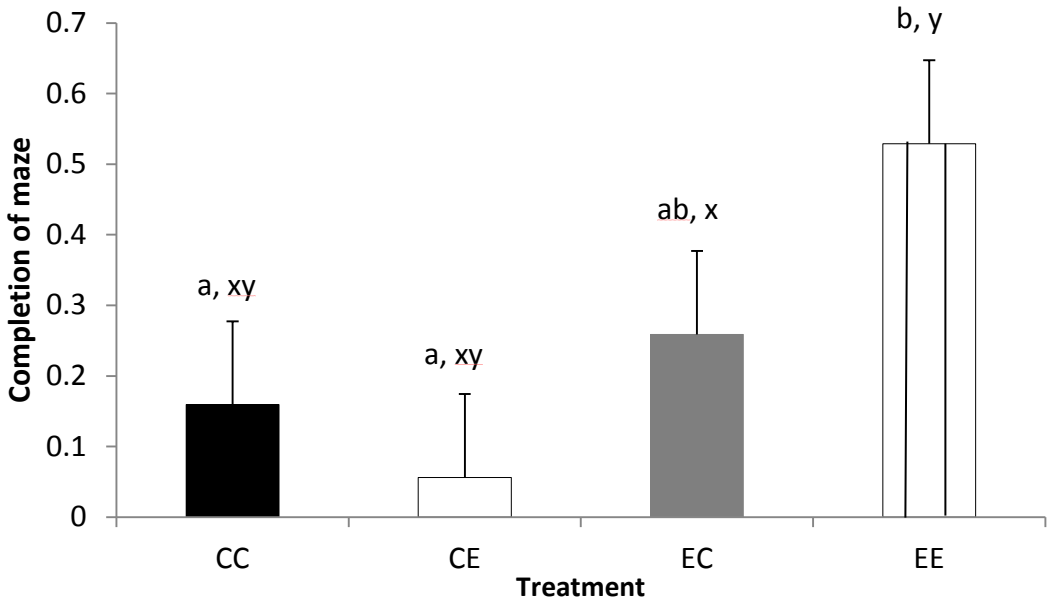

Figure 18. Number of maze II completions in experiment 2. Data are presented as LSMeans ± SEM for control-control (CC; black bars), control-ethanol (CE; white bars), ethanol-control (EC; grey bars), and ethanol-ethanol (EE; lined bars). Means without a common subscript at the beginning of the alphabet significantly differ at $P < 0.05$, and at the end of the alphabet tend to differ at $P < 0.10$.

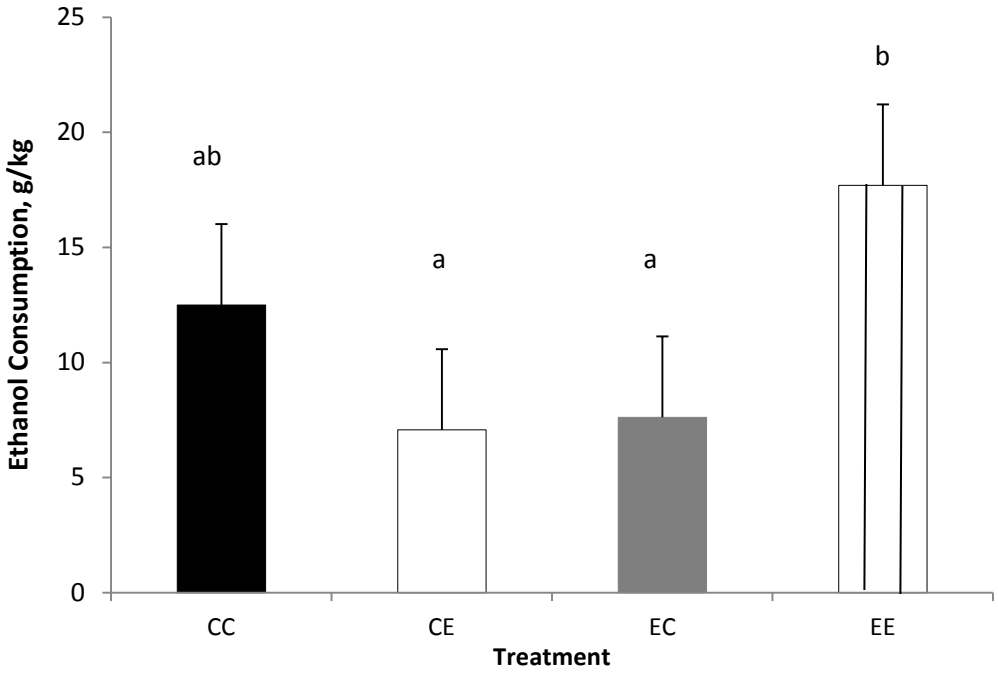

Figure 19. Amount of ethanol consumed at adolescence. Data are presented as LSMeans ± SEM for control-control (CC; black bars), control-ethanol (CE; white bars), ethanol-control (EC; grey bars) and ethanol-ethanol (EE; lined bars) pigs. Means without common subscripts differ at $P < 0.05$.

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CHAPTER IV

CONCLUSIONS

In Chapter II we found that 3 g/kg of 30% ethanol given to sows by intragastric gavage produced blood ethanol concentrations (BEC) above the legal limit in humans. This is the first step for an animal model to be a good alcohol model. However, the variability between sows was great so that the pharmacokinetics of ethanol in pregnant sows showed a long plateau phase and no distinct absorption then elimination phase. Not all sows had started the elimination phase at the end of the blood collection period, so a longer collection time is needed to establish the ethanol pharmacokinetic profile of pregnant sows.

More animals are needed to reduce the variation observed in this study. Other investigators have used intravenous methods of ethanol administration in young pigs to determine that ethanol metabolism is similar between pigs and humans. So that method is an option for repeated studies, however the size of the sows in our study required a large volume that may be difficult to deliver intravenously. We also chose intragastric gavage to produce the normal absorption, distribution and elimination that better represents the free choice drinking model we used in Chapter III and normal human consumption. We know from previous investigators that ethanol elimination in pigs is similar to humans, which makes the pig a good model for alcohol research, but more research is needed to determine ethanol pharmacokinetics of pregnant pigs for Fetal Alcohol Spectrum Disorder (FASD) research.

In chapter III, we found behavioral differences in anxiety, locomotion, social status, and learning between pigs from control sows and from sows given free access to 20% ethanol the entire length of gestation. We did not find any prenatal treatment effects on offspring weight or immune measures. And treatment effects on cortisol and immune measures were not found in sows, but the period of gestation did affect both. There were no differences in litter sizes or mortality, but ETOH sows did weigh more at the end of gestation compared to CON pigs which is different than most models of gestation drinking that show ethanol consumption during pregnancy decreases weight. Sows drank the most during the beginning and middle of gestation and decreased lower than a 2 g/kg average at the end of gestation.

In an isolation test we found CON pigs were more active compared to ETOH in both experiments, which may suggest ETOH pigs were less fearful of the environmental change. Increased activity during isolation may reflect an aggressive coping mechanism to a psychological stressor by actively trying to remove themselves from the environment. We did observe in experiment 1 that CON pigs were high responders to stress while ETOH low responders, but in experiment 2, it was EE pigs that had the higher number of high responders followed by CC pigs. The passive coping styles characteristic to ETOH pigs in experiment 1 may have allowed them to have a more flexible, adaptable response that is helpful in unfamiliar environments.

In the open field test, CON pigs in experiment 1 and EC pigs in experiment 2 showed more fear of the novel environment and object because they were less explorative and spent more time in the area they entered/exited the arena from. However, the open field test may be a better assessment of activity. Ethanol pigs in

experiment 1 were more active which is consistent with diagnosis of FASD offspring who are hyperactive and show signs of Attention Deficit/Hyperactive Disorder, but in experiment 2 CE pigs were more active. Control-Ethanol pigs in experiment 2 were also more active in the isolation test. Similar behaviors across both isolation and open field tests may indicate that CE pigs are more anxious and "restless."

In experiment 2, gestation treatment ETOH pigs tended to be dominant within the lactation litter by holding more cranial teats during nursing, and were dominant in social interaction tests with unfamiliar pigs. Increased aggression and willingness to seek out strangers is a characteristic of FASD, and could explain the higher social status observed in ETOH pigs in experiment 2 at younger ages. However, in both experiments during adolescence the social status within the home pen changed and ETOH pigs were move submissive to CON pigs in the food competition test. Social skill deficits become more prominent with age and FASD individuals may have problems establishing and maintaining relationships because social recognition and communication are impaired.

We found that ETOH pigs were quicker to complete mazes and more pigs completed the mazes compared to CON pigs. In experiment 2, lactation ETOH treatment pigs made the most wrong turns, but it was only significant for Maze I Memory. This is consistent with other investigators that found PAE rats completed different mazes more quickly even though they did make more errors. Increased activity could be one reason ETOH pigs were quicker to reach the reward, however more ETOH pigs did successfully complete the maze compared to CON pigs which

suggests they did learn the maze and did not require more repetitions. Learning in PAE pigs was the same if not better than CON pigs.

Some changes and deficits characteristic of FASD may not be seen until later in life or during stressed conditions. Prenatal exposure to ethanol increases the responsiveness of ethanol, and PAE offspring may be predisposed to increased alcohol abuse during adolescence when stress is high. In the pig model, we found EE pigs drank the most ethanol during individual but not group housed conditions. When animals are stimulated by environmental or social enrichment, ethanol consumption does decrease, so the anxiolytic effects of ethanol may be more prominent in isolated conditions when anxiety and/or depression is greatest.

The domestic pig model for FASD is novel research. Pigs are very comparable to humans in anatomy, physiology, and behavior making them a great in biomedical research. They are also highly intelligent, social animals and can be used in behavioral tests adapted from rodent studies. These similarities between humans may make extrapolating animal model findings to humans easier. However, this is new research and more studies and animals are needed. We chose a free choice drinking paradigm because antidotal stories talk about how they will drink to drunkenness voluntarily. We can assume in our study fetuses were most affected during early and mid development, but without a more controlled drinking model it is hard to know exactly how much ethanol the sow consumed and if it was enough to cross the placenta and affect fetal development.

There is a spectrum of consequences in maternal gestational drinking; with only taking one day of peak BECs to have long term consequences on the offspring,

and 2 g/kg consumption is a moderate level that has been known to affect offspring. So it is likely our results are from prenatal exposure to ethanol and not just the variability of animals. But the individual pig variability in behavioral results in our study could be from not every pig receiving the same dose of ethanol prenatally. This study does suggest that behavior changes can result from the teratogenic effects of ethanol in a pig model; however a larger sample size is needed to bring some of our tendencies to a level of significance.

We need to do more basic research in the pig model, starting first with establishing the drinking paradigm in the sow. We should conduct studies to establish how much ethanol is needed for peak BECs in the sow, the best place for blood collection, BEC levels required to cross the placenta, and then look at different stages of gestation on fetal development. Investigators also dispute the most sensitive time of exposure, so after the stages of pig gestation are correlated to humans then future studies would focus on affecting specific periods of development or organs, with continuous or a single bolus of ethanol. Once we have conducted studies giving a set amount of ethanol to sows we then need to address different methods of free choice consumption, starting with preferences and finding ways to get sows to drinking high amounts of ethanol voluntarily for long periods of time with methods such as sucrose fading or slowly increasing concentrations. After the pig model is expanded upon, then we can look at the effects of the offspring developmentally, physiologically, and behaviorally.

We did find that sows can become intoxicated and high levels of BECs are measurable, and that behavioral responses of pigs from ETOH consuming sows

differed. Increased activity and aggressiveness at an early age, with changes in social skills at adolescence and increased drinking seen in our study are characteristic of FASD. So the pig may be a good model for FASD behavioral research.