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Modeling Ultrasonic Vocalization Profiles As Predictive Biomarkers For Alcohol Consumption Susceptibility

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Modeling Ultrasonic Vocalization Profiles As Predictive Biomarkers For Alcohol Consumption Susceptibility

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Dedication

This dissertation is dedicated to my dear bauji (Kharaiti Lal) for always believing in me, and to my parents (Simmi and Gurinder) for their hard work, unwavering devotion, ongoing support, and unconditional love.

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Modeling Ultrasonic Vocalization Profiles As Predictive Biomarkers For Alcohol Consumption Susceptibility

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The overarching goal of this dissertation project is to characterize the ultrasonic vocalization profiles associated with sex differences and predisposition for alcohol consumption in rodents. In order to accomplish this goal we pursued the following specific aims: 1) to investigate potential sex differences in the USV profiles of 50 – 55 kHz frequency modulated (FM) and 22 – 28 kHz calls in male and female high-alcohol-drinking (HAD-1) rats, 2) to investigate potential differences in the counts and acoustic characteristics of 50 – 55 kHz FM and 22 – 28 kHz USVs between HAD-1 and low-alcohol-drinking (LAD-1) rats, and 3) to determine whether USV profiles can serve as biomarkers for the predisposition to consume high levels of alcohol in Long-Evans rats.

The results from the studies assessing the first aim are described in chapters 2 and 3. We found that male and female HAD-1 rats spontaneously emit large amounts of unprovoked 50 – 55 kHz FM and 22 – 28 kHz USVs with distinct acoustic properties, which are further susceptible to modulation by ethanol. We also found that female HAD-1 rats show enhanced exploratory activity in an object recognition task, and that these exploratory behaviors predict future alcohol consumption levels in male and female HAD-1 rats. The results for the second aim are described in chapter 4, where we show that USV acoustic profiles can be used to generate machine learning classification

models, which can discriminate between HAD-1 and LAD-1 rats with very high accuracy.

The results for the third aim are described in chapter 5, where we show that USV data from alcohol-naïve rats across five different rat lines is significantly correlated with predisposition alcohol consumption. Here we provide direct evidence that USV data collected from alcohol-naïve, adult, male Long-Evans rats can be used to predict future alcohol consumption in these rats. In chapter 6, we further characterize changes in the total counts and acoustic characteristics of spontaneously emitted USVs due to age and ethanol exposure in adult, male Long-Evans rats. We finish in chapter 7 by summarizing the results and discussing the implications, as well as, potential future directions of this work.

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Chapter 1: Introduction

HISTORY OF ALCOHOL USE

Alcohol is one of the oldest drugs of abuse known to humankind. For over 10,000 years alcoholic beverages have been a popular source of hydration and calories for human beings. The discovery of alcohol was likely serendipitous with some stone age character accidentally experiencing a jar of honey or fruit gone bad through the natural process of fermentation. The curious oenologist, intrigued by the intoxicating effects of alcohol, probably replicated the process, which was eventually codified and passed down to the future generations. Indeed, there are 6000-year-old Babylonian tablets containing recipes for beer and 4000-year-old Egyptian hieroglyphs depicting inebriated revelers being carried off after a banquet (Vallee, 1998).

Although the risks associated with excess alcohol consumption were recognized even at that time, they likely paled in comparison to the risks associated with drinking water from a contaminated source. The antiseptic and acidic properties of alcohol allowed it to be non-perishable and relatively pathogen free source of hydration. Due to the lack of safer alternatives criticisms of alcohol were likely very limited. With natural fermentation only producing alcoholic beverages with a maximum concentration of approximately ~16%, the magnitude of alcohol consumption remained somewhat restricted until the development of the distillation process by Arab chemists around 700 A.D. (Vallee, 1998).

Distillation allowed for the production of beverages with very high concentrations of alcohol, exposing people to an unprecedented level of inebriation. Over the next few

centuries the knowledge of distillation spread throughout the European society. The first printed book on distillation *Liber de arte distillandi* published in 1500 became a best-seller. It wasn't until the introduction of beverages made with boiled water, such as coffee and tea, that alcohol's monopoly as a safe drinking choice was threatened (Vallee, 1998).

The availability of safer alternatives, allowed religious sentiments against the morality of alcohol to gain ground. Finally, with the advent and application of scientific principles in medicine, alcohol abuse became one of the first medical problems to be recognized as a chronic, life-threatening disease. Thomas Trotter and Benjamin Rush published essays in the early 1800's describing that the habitual and prolonged consumption of alcohol lead to liver disease, including jaundice, wasting and mental dysfunction, even when the patients were sober (Vallee, 1998). In the United States, alcohol consumption has been steadily rising since 1850, with the exception of the period of prohibition. According to a 2013 survey nearly ~52% of Americans over the age of 12 reported being current drinkers of alcohol, nearly ~22% reported having a binge episode within the past month, and approximately ~6.3% reported engaging in heavy drinking (Substance Abuse and Mental Health Services Administration and Administration, 2014). Although alcohol is a widely accepted 'social lubricant', its excessive use produces more harm to society than all other drugs of abuse.

NEUROCHEMICAL SUBSTRATES UNDERLYING ALCOHOL USE AND ABUSE

Many of the primary neurochemical systems play a significant role in the rewarding and reinforcing properties of alcohol, including gamma-aminobutyric acid (GABA) (Harris and Allan, 1989), glutamate (Goodwani et al., 2017), dopamine

(Trantham-Davidson and Chandler, 2015), opioid (Modesto-Lowe and Fritz, 2005), serotonin (Marcinkiewcz et al., 2016) and acetylcholine (Rahman et al., 2016) systems.

GABA

GABA receptors are the primary inhibitory receptors in the brain. Acute intoxication of alcohol produces anxiolysis, sedation and impairment in motor and cognitive functions. The acute intoxicating effects of alcohol are mediated through the activation of $GABA_A$ receptors either by direct-binding or the indirect release of GABA or GABAergic steroids (Lobo and Harris, 2008; Most et al., 2014). Similar effects can be achieved either with GABA agonists, such as muscimol, or GABA positive allosteric modulators, such as benzodiazepines (Martz et al., 1983). Conversely, antagonists or inverse agonists of GABA can reduce the motor-impairing (Hellevuo et al., 1989) and anxiolytic effects of alcohol (Liljequist and Engel, 1984), as well as reduce alcohol self-administration (Samson and Chappell, 2001). Alcohol administration also increases signaling of the metabotropic $GABA_B$ receptors (Federici et al., 2009) and $GABA_B$ receptor agonist baclofen has been shown to decrease alcohol self-administration (Janak and Gill, 2003) and alcohol deprivation effect in rats (Colombo et al., 2003). Clinical studies on the efficacy of baclofen in treating alcoholics however, have produced mixed results with some studies showing baclofen suppressing alcohol-withdrawal symptoms and promoting abstinence (Addolorato et al., 2002), while others show no such effect (Garbutt et al., 2010).

Glutamate

Glutamate is the primary excitatory neurotransmitter in the central nervous system. The glutamatergic system includes both metabotropic and ionotropic glutamate

receptors. The primary ionotropic glutamatergic receptors in the brain are N-methyl D-aspartate (NMDA) and a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Acute alcohol inhibits both AMPA (Dildy-Mayfield and Harris, 1992, 1995) and NMDA (Lovinger et al., 1989) receptor function, while chronic alcohol use up-regulates AMPA (Lewohl et al., 2000) and NMDA (Qiang and Ticku, 2005) receptor expression in the brain. NMDA antagonists MK-801 and ketamine can mimic the behavioral (Krystal et al., 1998) and subjective (Butelman et al., 1993) effects of alcohol. Metabotropic glutamate receptors are separated into group 1, which contain metabotropic glutamate receptors 1 and 5 (mGluR1 and mGlurR5), group 2, containing receptors 2 and 3 (mGluR2 and mGluR3), and group 3, containing (mGluR4, mGluR6, mGluR7 and mGluR8). mGluR5 receptor blockers 2-methyl-6-(phenylethynyl)pyridine (MPEP) and Acamprosate have been shown to attenuate alcohol-seeking behaviors (Blednov and Harris, 2008). Studies have also shown that mGluR2/3 agonist LY379268 can reduce alcohol self-administration behaviors and cue-induced alcohol seeking (Sidhpura et al., 2010). Mutations resulting in decreased expression of mGluR7 are associated with increased alcohol consumption (Vadasz et al., 2007). Moreover, mice lacking mGluR4 do not show alcohol-induced stimulation of motor activity (Blednov et al., 2004).

Dopamine

The mesolimbic dopaminergic system plays an important role in the natural and drug-use related reward and reinforcement (Volkow and Morales, 2015). Dopamine is thought to modulate the effects of alcohol through its modulation of dopaminergic signaling (Gonzales et al., 2004). Alcohol and other drugs of abuse increase midbrain dopaminergic activity in humans and rodents alike (Budygin et al., 2001; Boileau et al., 2003). Specifically, alcohol self-administration has been shown to increase extracellular

levels of dopamine in the nucleus accumbens (Weiss et al., 1993). Moreover, systemic injections of dopamine have been shown to decrease responding for alcohol (Pfeffer and Samson, 1986; Rassnick et al., 1992). Intra-accumbal injections of the dopaminergic antagonist fluphenazine block alcohol self-administration in rodents (Rassnick et al., 1992). Moreover, the antipsychotic dopaminergic antagonist aripiprazole has also been shown to reduce cravings and increase positive feelings in human alcoholics (Martinotti et al., 2007; Brunetti et al., 2012). While acute alcohol exposure increases dopamine transmission to promote reward (Robinson et al., 2009; Schier et al., 2013), chronic alcohol use results in allostatic changes which can produce a hypodopaminergic state and result in anhedonia, aversion and blunted reward processing associated with alcohol withdrawal (Volkow et al., 2004; Koob and Volkow, 2010; Koob, 2015).

Opioid

The release of opioid peptides has long been hypothesized to play a role in producing the reinforcing effects of alcohol (Herz, 1997). The opioidergic system consists of three primary classes of opioid receptors: mu, kappa and delta. Opioid receptors are found presynaptically in dopaminergic neurons and regulate dopaminergic activity in response to alcohol and other drugs of abuse. Variations in opioid-related gene expression and function are associated with high levels of alcohol consumption (Marini et al., 2013). Studies have shown that low doses of opioid agonists can increase alcohol consumption, while high doses of opioid agonists result in a decrease in consumption levels (Modesto-Lowe and Fritz, 2005). These findings suggest that small doses of opioid agonists might serve as ‘appetizers’ that increase the drive for alcohol consumption, while high doses might serve to produce sufficient reward resulting in a decrease in the drive for alcohol. In line with these results, the opioid antagonist naltrexone has been

shown to reduce alcohol consumption in both rodents and humans and in preventing relapse to alcohol (Davidson and Amit, 1997).

Serotonin

Serotonin plays an important role in regulating mood, sleep, appetite, learning and memory. Serotonin levels in the brain are elevated after acute alcohol exposure (Murphy et al., 1982). Alcohol facilitates serotonergic transmission by increasing the potency (Sung et al., 2000) and open time of serotonin-3 (5HT-3) receptor (Zhou et al., 1998). 5HT-3 receptor antagonists decrease alcohol self-administration in rodents (Sellers et al., 1994), and also decrease drinks-per-day, while increasing abstinence time in human alcoholics (Johnson et al., 1993, 2011). There is thought to be an inverse relationship between serotonin transmission and alcohol consumption, with increased serotonin transmission being associated with lower levels of alcohol consumption and decreased transmission resulting in increased consumption in rodent models and humans alike (Lovinger, 1997). Selective serotonin reuptake inhibitors have shown some clinical efficacy in reducing alcohol consumption in human alcoholics (Naranjo and Knoke, 2001).

Acetylcholine

Acetylcholine was one of the first neurotransmitters to be discovered. Cholinergic transmission is known to play an important role in mediating arousal, attention, memory and motivation. Acetylcholine transmission in the brain occurs either through projection neurons or local interneurons in a range of cortical and subcortical structures. The two primary cholinergic receptor subtypes are the muscarinic and nicotinic receptors. Cholinergic activation of both the muscarinic and nicotinic acetylcholine receptor

subtypes plays a critical role in modulating dopaminergic activity in the ventral tegmental area (Fagen et al., 2003; Picciotto et al., 2012). The dopamine activating and reinforcing properties of ethanol are in part mediated through the activation of nicotinic acetylcholine receptors in the ventral tegmental area (Ericson et al., 2003; Löf et al., 2007; Wu et al., 2014). Moreover, prolonged alcohol use is associated with cholinergic dysfunction, decreased cortical acetyl choline and a reduction in choline acetyltransferase-positive neurons (Ehrlich et al., 2012). Cholinergic antagonist mecamylamine and partial agonist varenicline have also been shown to reduce drinking behaviors in both rodents and human alcoholics (Ericson et al., 1998; Erwin and Slaton, 2014). Furthermore variations in genes encoding both the muscarinic (Wang et al., 2004) and nicotinic (Wang et al., 2009) cholinergic receptor subtypes is associated with alcohol abuse and dependence.

EMOTIONAL STATUS MODULATES ALCOHOL AND DRUG USE

Drug addiction has been described a chronic relapsing disorder with three stages (1) described as a compulsion to seek and take the drug, (2) a loss of control in limiting intake, and (3) the emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability) when the drug is no longer available (Koob and Volkow, 2016). The above definition implies a unidirectional relationship whereby drugs of abuse can create a pathological state of emotional dysregulation, which can in turn perpetuate increased drug use. However, there is mounting evidence in support of a bidirectional relationship where emotional dysregulation and heightened negative emotionality, brought on by external factors, such as depression (Schuckit et al., 2006; Conner et al., 2009), traumatic stress (Weiss et al., 2015), and adversity (Sorocco et al., 2015) can increase the risk of developing a substance use disorders (SUD).

Individuals that exhibit a difficulty in the ability to regulate their emotional state are more likely to engage in 'drinking to cope' behaviors and subsequently transition towards hazardous alcohol consumption (Watkins et al., 2015; Pombo et al., 2016). Moreover, interventions aimed at improving emotional states via mindfulness and meditation have been shown to decrease craving for drugs of abuse (Tang et al., 2016). The neural structures involved in the regulation of emotion include: amygdala, basal forebrain, brainstem, hypothalamus, and the ventromedial prefrontal and the cingulate cortices (Koob, 2015). Basal emotional states serve as a functional reflection of the activity of these brain regions and dysregulation of function in these regions is an important component of drug abuse and addiction (Koob and Volkow, 2016). Thus, it is not surprising that a pre-existing state of heightened negative affect and emotional dysregulation can lead to the emergence and maintenance of pathological drug use.

EMOTIONAL AND SUBJECTIVE RESPONSES TO ALCOHOL PREDICT CONSUMPTION

Acute emotional and subjective responses to alcohol have also been identified as a critical phenotypic risk factor for the development of alcohol use disorders (AUD). Currently, two prevalent, but contradictory, clinical models exist to explain the role of subjective responses to alcohol. The Low Level of Response Model (LLRM) contends that individuals with high risk for alcohol use experience lower subjective response to alcohol relative to individuals with a low risk. The Differentiator Model (DM) proposes that individuals at risk of AUDs may be more susceptible to the euphoric effects of alcohol that occur during the rising phase of blood alcohol levels, and less sensitive to the sedative effects of alcohol that occur during the falling phase of blood alcohol levels. Current literature has been divisive on this topic, as studies have provided partial

evidence for both models (Morean and Corbin, 2010; Quinn and Fromme, 2011; Ray et al., 2016). While subjective response literature has become an important aspect of many clinical studies in the alcohol abuse field, the relationship between subjective responses and alcohol consumption in preclinical studies remains unknown.

THE NEED FOR STUDYING EMOTION AND ALCOHOL USE IN A PRECLINICAL MODEL

The findings above highlight a clear need for understanding the causal relationship between emotional states and the onset of SUDs. Unfortunately, most of the studies exploring the role of emotional and subjective responses to alcohol in the development of AUDs have been limited to clinical populations, which do not provide direct causal evidence. Studies investigating the causal relationships between the various neural substrates of drug abuse are typically conducted in preclinical rodent models, which can utilize a variety of experimental manipulations not available to clinical investigators (Rosenwasser, 2015). However, this has been difficult to achieve due to a lack of valid objective measures of emotionality in rodents.

SELECTIVELY-BRED RAT LINES AS PRECLINICAL MODELS OF ALCOHOLISM AND ALCOHOL ABUSE

The development of high alcohol-consuming rat lines through selective breeding has been important in identifying genetic factors that underlie inter-individual differences in alcohol use and abuse. Examples of such lines include alcohol-preferring (P) rats and high alcohol-drinking (HAD) rats, as well as, their counterparts the alcohol non-preferring (NP) rats and low alcohol-drinking (LAD) rats. These lines are widely used by

laboratories around the world and fit many of the criteria for an animal model of AUDs (Rodd et al., 2004; Bell et al., 2016):

1. Voluntary oral self-administration of 5 – 8 g/kg ethanol under free drinking conditions (McBride and Li, 1998; Murphy et al., 2002).
2. Self-administration of ethanol results in pharmacologically relevant BECs (50 – 200 mg%) (McBride and Li, 1998; Murphy et al., 2002).
3. Ethanol is consumed primarily for its intoxicating effects and not for its taste, smell or caloric value (Toalston et al., 2008; Russell et al., 2011).
4. Operant responding for ethanol produced BECs in excess of 80 mg% and these rats display high breakpoints during progressive ratio testing (Rodd et al., 2003; Oster et al., 2006).
5. Chronic ethanol consumption leads to metabolic and functional tolerance to the motor-impairing and aversive effects of ethanol (Lumeng and Ting-Kai, 1986; Gatto et al., 1987).
6. Exhibition of an alcohol deprivation effect and physical signs of withdrawal following withdrawal from chronic ethanol consumption (Waller et al., 1982; Kampov-Polevoy et al., 2000).

In line with the human literature on genetic variation in neurotransmitter systems, the high alcohol-consuming rats also show clear differences in many neurotransmitter systems when compared to their low drinking counterparts; including the dopaminergic, GABAergic, serotonergic, cholinergic and opioidergic systems (Murphy et al., 2002; Bell et al., 2012, 2016). Research in our lab has focused on dopaminergic and cholinergic transmission in two pairs of these selectively bred rat lines, namely the P/NP and the HAD/LAD rats. Both these lines are known to have basal differences in dopaminergic

and cholinergic transmission. For instance, P and HAD rats have lower tissue levels of DA and its metabolites (DOPAC and HVA) in the nucleus accumbens and the anterior striatum as compared to their NP and LAD counterparts (Murphy et al., 1982, 1987; Gongwer et al., 1989). This may be due to a potential deficit in the VTA DA projections in these rat lines. Indeed P rats have reduced DA projections from the VTA to the nucleus accumbens (Zhou et al., 1995), as well as, lower levels of DA D_2 receptor binding sites (McBride et al., 1993), as compared to the NP rats. In order to compensate for the lower levels of DA, P rats show increased burst firing of VTA DA neurons, though a similar increase was not seen in the HAD rats (Morzorati, 1998; Morzorati and Marunde, 2006). Alcohol consumption results in reduced D_2 auto-receptor function (Engleman et al., 2000, 2003), and enhanced DA efflux (Weiss et al., 1993; Thielen et al., 2004) and reuptake (Sahr et al., 2004).

The potential differences in cholinergic transmission between the selectively bred rat lines remain largely unexplored. However, the few studies that have been conducted suggest that the accumbal cholinergic system may be more active in P rats than NP rats (Bell et al., 2016). For instance, P rats have lower expression levels of striatal nicotinic acetylcholine receptors than NP rats (Tizabi et al., 2001). Studies have also identified higher expression levels of genes encoding choline acetyltransferase, muscarininc acetylcholine receptor 3, and channels responsible for the uptake and vesicular transport of acetylcholine during synthesis in the nucleus accumbens shell of P rats as compared to the NP rats (McBride et al., 2013a, 2013b). Furthermore, administration of nicotinic antagonists or desensitizing agents can reduce alcohol consumption in P rats (Rezvani et al., 2010; Srisontiyakul et al., 2016). Together, these results show that selective breeding for alcohol preference results in clear differences in dopaminergic and cholinergic

systems and highlight the important role of DA and ACh transmission in alcohol consumption.

Selective breeding for alcohol consumption provides a powerful tool for the identification of genetic, and in turn phenotypic, traits correlated with high and low levels of alcohol consumption (Crabbe, 2008; Bell et al., 2012). However, due to the small populations typically used for creating a selected line (i.e. rats with the highest or the lowest levels of alcohol consumption), it is possible for inbreeding to occur. This inbreeding can, in turn, lead to the generation of potentially random differences, due to genetic drift, between the selectively-bred lines (Crabbe et al., 1990; Grahame, 2000). In order to overcome this limitation, it is important to ensure that alcohol associated phenotypes are observed in a variety of selectively bred lines. Alcohol associated traits seen in selectively-bred lines should also be generalizable to other outbred strains, such as the Long-Evans (LE) and Wistar rats, which are commonly used in preclinical alcohol research. These outbred strains can be particularly important in investigating inter-individual variability in alcohol consumption, which is observed in both humans and rodents alike.

ULTRASONIC VOCALIZATIONS AS A REAL-TIME MEASURE OF EMOTIONAL STATUS IN RODENTS

Spontaneous ultrasonic vocalizations (USVs) are emitted by rodents and considered to be indices of anticipatory affective state of the animal. Converging evidence from ethological, pharmacological, and neuroanatomical studies has shown that 22-28 kHz USVs represent a negative affective state and occur in response to alarm, punishment, or avoidance behaviors; while 50-55 kHz USVs represent positive affect and

are produced in response to rewarding stimuli including food, drugs, or sex (Knutson et al., 2002; Brudzynski, 2013). Although these calls are inaudible to the human ear, they can be recorded using specialized equipment and can serve as a real-time measure of the affective state of a rat.

ULTRASONIC VOCALIZATIONS AS BIOMARKERS OF DOPAMINERGIC AND CHOLINERGIC TRANSMISSION

Ultrasonic vocalizations (USVs) are an established, non-invasive, real-time, functional biomarker of dopaminergic and cholinergic transmission in rodents (Brudzynski, 2015). USVs are typically separated into two common subtypes: 50 – 55 kHz frequency modulated (FM) USVs, which are associated with positive affective states and rewarding stimuli (Burgdorf and Panksepp, 2006; Ciucci et al., 2009; Buck et al., 2014b; Popik et al., 2014; Avvisati et al., 2016; Brenes et al., 2016; Roccaro-Waldmeyer et al., 2016; Williams and Undieh, 2016) and 22 – 28 kHz USVs, which are associated with stressful, traumatic and alarming situations (Brudzynski and Bihari, 1990; Lindquist et al., 2004; Wang et al., 2008; Bardin et al., 2010; Chen et al., 2012; Parsana et al., 2012; Berger et al., 2013; Furlanetti et al., 2015).

50 – 55 kHz FM USVs

50 – 55 kHz FM USVs can be directly elicited through the activation of dopaminergic transmission via the administration of psychostimulants such a cocaine, amphetamine, or methylphenidate (Burgdorf et al., 2001; Ahrens et al., 2009; Maier et al., 2012). Moreover, these types of calls can be suppressed through the antagonism or degradation of the nigrostriatal dopaminergic pathway (Wintink and Brudzynski, 2001;

Ciucci et al., 2009; Wright et al., 2013). Studies have also shown that acoustic characteristics, such as the mean frequency and bandwidth, of these calls can also be modulated through pharmacological manipulations of dopaminergic transmission (Brudzynski et al., 2011; Simola, 2015).

22 – 28 kHz USVs

22 – 28 kHz USVs can be directly elicited via cholinergic activation of the medial hypothalamic/preoptic region in rats or via the administration of cholinergic agonists, such as carbachol (Brudzynski and Bihari, 1990; Brudzynski et al., 1993). Conversely, these USV subtype calls can be antagonized through the application of cholinergic antagonists, such as atropine and scopolamine (Brudzynski, 2001). Specific characteristics of 22 – 28 kHz USVs, such as call duration, power, and bandwidth, can also be modulated by cholinergic agonists in a dose dependent manner (Brudzynski, 1994).

Since these pathways are known to be affected by both acute and chronic alcohol intake (Muller et al., 1980; Ericson et al., 2010), USVs may be used as a proxy or biomarker to study underlying neural differences in high and low alcohol consuming rats. Indeed, recent studies in our laboratory have shown that rats selectively bred for high alcohol drinking behavior (P and HAD rats) spontaneously emit negative affect USVs that are associated with alcohol availability status and alcohol consumption (Reno et al., 2015; Thakore et al., 2016). Further, our laboratory has recently shown that alcohol-preferring (P) rats can be distinguished from alcohol non-preferring (NP) rats simply from the properties of their 22-28 kHz USVs (Reno et al., 2017), suggesting a regulatory role of negative affect in alcohol consumption levels.

LIMITATIONS OF MANUAL ULTRASONIC VOCALIZATION ANALYSIS TECHNIQUES.

Although USVs can serve as non-invasive, functional biomarkers of neurotransmission, they are not widely used for this purpose due to the extensive time and effort required to manual score USV data. Manual analysis of USVs requires the experimenter to play back the recorded audio files at 4% of the original speed and acoustically identify each call. As an example, manual analysis of a 10-minute USV file would take approximately 4 hours to complete. Because of this, nearly all USV studies in the past have focused on short recording periods (e.g., less than 30 minutes) and reported very basic measures such as total call counts (Armstrong et al., 2001; Burgdorf et al., 2007; Portfors, 2007; Scattoni et al., 2009; Brudzynski, 2015; Johnson et al., 2015; Kaniuga et al., 2016). In order to overcome this bottleneck in manual USV analysis there has been recent interest in the development of automated USV analysis tools. These include the WAAVES algorithm developed in our lab (Reno et al., 2013; Reno and Duvauchelle, 2014) and a template-matching technique developed by Barker and colleagues using XBAT (Barker et al., 2014).

WAV-FILE AUTOMATED ANALYSIS OF VOCALIZATIONS ENVIRONMENT SPECIFIC (WAAVES)

WAAVES is a highly accurate algorithm that automates the analysis of USVs (Reno et al., 2013; Reno and Duvauchelle, 2014). This algorithm has made it possible for us to conduct experiments with relatively long timelines, something that was previously not feasible. This program reads audio files and produces a frequency spectrogram. The spectrogram is then scanned for sound objects using MATLAB's *Image Processing Toolbox* (MathWorks, Inc. Natick, MA). A series of filters are applied to separate the

background noise from USV calls. Once the calls are identified, several measurements of interest are extracted from each USV call and stored for subsequent analysis. WAAVES also allows us to examine each USV in much greater detail. In addition to the ability to count USVs, WAAVES allows us to calculate a number of acoustic characteristics associated with each USV. These include the call mean frequency in kilohertz, call duration in milliseconds, call bandwidth in kilohertz, and call power in decibel. The result is a rich, multi-dimensional representation of each unique USV. These can be combined into large data sets that can be analyzed using advanced statistical models and machine learning algorithms in order to explore the intricate relationships between rat USV characteristics and behavioral phenotypes, such as alcohol seeking and consumption.

Chapter 2: Sex-specific ultrasonic vocalization patterns and alcohol consumption in high alcohol-drinking (HAD-1) rats [1]

ABSTRACT

Ultrasonic vocalizations (USVs) have been established as an animal model of emotional status and are often utilized in drug abuse studies as motivational and emotional indices. Further USV functionality has been demonstrated in our recent work showing accurate identification of selectively-bred high versus low alcohol-consuming male rats ascertained exclusively from 22 – 28 kHz and 50 – 55 kHz FM USV acoustic parameters. With the hypothesis that alcohol-sensitive sex differences could be revealed through USV acoustic parameters, the present study examined USVs and alcohol consumption in male and female selectively bred high-alcohol drinking (HAD-1) rats. For the current study, we examined USV data collected during a 12-week experiment in male and female HAD-1 rats. Experimental phases included Baseline (2 wks), 4-hr EtOH Access (4 wks), 24-hr EtOH Access (4 wks) and Abstinence (2 wks). Findings showed that both male and female HAD-1 rats spontaneously emitted a large number of 22 – 28 kHz and 50 – 55 kHz FM USVs and that females drank significantly more alcohol compared to males over the entire course of the experiment. Analyses of USV acoustic characteristics (i.e. mean frequency, duration, bandwidth and power) revealed distinct sex-specific phenotypes in both 50 – 55 kHz FM and 22 – 28 kHz USV transmission that were modulated by ethanol exposure. Moreover, by using a linear combination of these acoustic characteristics, we were able to develop binomial logistic regression models able to discriminate between male and female HAD-1 rats with high accuracy. Together these

[1] Reprinted from Mittal, N., Thakore, N., Bell, R. L., Maddox, W. T., Schallert, T., & Duvauchelle, C. L. (Copyright 2017). Sex-specific ultrasonic vocalization patterns and alcohol consumption in high alcohol-drinking (HAD-1) rats. Physiology & Behavior. https://doi.org/10.1016/j.physbeh.2017.11.012, with permission from Elsevier. NM was responsible for experiment design, data collection, analysis, interpretation, and writing of this manuscript.

results highlight unique emotional phenotypes in male and female HAD-1 rats that are differentially modulated by alcohol experience.

INTRODUCTION

Alcohol research has focused almost exclusively on males in both clinical and preclinical investigations. However, to fully understand alcoholism it is necessary to study mechanistic differences across sexes. Though historically, men consume more alcohol than women, recent data suggest that men and women are becoming more similar in alcohol abuse patterns (Substance Abuse and Mental Health Services Administration and Administration, 2014), such as alcohol drinking frequency and driving while intoxicated (White et al., 2015). In addition, research suggests that women are more vulnerable than men to the brain-damaging effects of alcohol, require shorter periods of excessive drinking before seeing negative effects (Hommer, 2003), and are more susceptible to the negative consequences of alcohol, such as cirrhosis, alcoholic liver injury and alcoholic cardiomyopathy (Fernández-Solà et al., 1997; Eagon, 2010; Erol and Karpyak, 2015).

A variety of factors, including pharmacokinetic differences in alcohol metabolism, play a role in the manifestation of sex difference in alcohol consumption behaviors. For instance, evidence suggests that females have greater alcohol clearance per unit lean body mass (Kwo et al., 1998) and enhanced first-pass metabolism of alcohol and its metabolites (Ward and Coutelle, 2003). Moreover, research indicates that alcohol may modulate hormonal levels and function through its effects on both the hypothalamus and the gonads (Devaud et al., 2006). Together these results highlight the need for an improved understanding of the complex interaction between biological factors and alcohol consumption that underlie sex differences seen in alcohol-associated behaviors.

However, unlike humans, female rats have often been reported to drink more than male rats (Almeida et al., 1998; Maldonado-Devincci et al., 2010; Nieto and Kosten, 2017). Therefore, it is necessary to consider various factors contributing to this dichotomy in sex-specific alcohol consumption behaviors between rodents and humans.

The High Alcohol-Drinking (HAD-1) rats were selectively bred from the heterogeneous N/NIH stock line for a preference of ethanol (10%, v/v) over water and meet nearly all of the criteria set forth for a suitable animal model of alcoholism (Lester and Freed, 1973; Gongwer et al., 1989). In the current study, we focused on the effects of sex in HAD-1 ultrasonic vocalization (USV) characteristics and alcohol use to examine sex-specific motivation and emotional components of alcohol consumption. We take advantage of a growing body of research indicating that ultrasonic vocalizations emitted by rats reflect real-time emotional status and the wide acceptance of USV indices as animal models of affect (Burgdorf and Panksepp, 2006; Brudzynski, 2013). For instance, rodents emit USVs in the 50 – 55 kHz and 22 – 28 kHz ranges that are reliably associated with positive and negative emotional states, respectively (Brudzynski et al., 1993; Knutson et al., 2002; Burgdorf and Panksepp, 2006). USVs have received increased attention in drug abuse studies because administration of cocaine (Maier et al., 2012), amphetamine (Ahrens et al., 2009) and drug-associated cues (Ma et al., 2010; Maier et al., 2010) increase 50 – 55 kHz frequency-modulated (FM) USV emissions. In addition, escalated levels of alcohol consumed by alcohol-dependent rats are significantly correlated with alcohol anticipatory 50 – 55 kHz FM USVs (Buck et al., 2014a) and alcohol-dependent rats in a withdrawal state are more easily provoked to emit negative affect-associated 22 – 28 kHz USVs by mild aversive stimuli (Berger et al., 2013). Recent studies in our laboratory have shown that selectively bred alcohol-naïve alcohol-preferring P rats and HAD-1 rats are unique in that they emit numerous spontaneous 22-

28 kHz USVs during baseline recordings in the absence of drug experience or external provocation (Thakore et al., 2016; Mittal et al., 2017b; Reno et al., 2017).

Previous studies examining acoustic characteristics have done so by averaging across all of the USVs of a given type emitted by each animal (Brudzynski et al., 1991; Brudzynski, 1994; Inagaki et al., 2012; Reno et al., 2015; Thakore et al., 2016). This allows for traditional statistical analyses (e.g. ANOVAs) to be employed by creating equal sample sizes across days. However, each USV is multi-dimensional and can be characterized by values along each of many acoustical dimensions (e.g., frequency, duration, bandwidth, power). Therefore, reducing the data to a single number of central tendency (e.g. mean) loses much of the data's intricacies. The linear mixed model (LMM) is similar to an analysis of variance (ANOVA), but LMM allows for unequal sample sizes and missing data points. Therefore, each individual USV is included in the analysis. This method allows the researcher to identify the best-fit model from the given predictors (e.g. rat line, alcohol experience, day). This model takes these highly variable data and reveals underlying patterns (Kuznetsova et al., 2016). While the linear mixed model (LMM) examines one acoustic characteristic at a time, binomial logistical regression (BLR) allows for the combined interactive effect of all four USV characteristics to calculate a probability value that represents the maximum separation between male and female groups.

The current study extends our previous work with HAD-1 rats in a drinking-in-the-dark (DID) paradigm (Thakore et al., 2016) by including male and female rats and by utilizing a more sophisticated experimental design. In this study, experimental sessions were conducted 4 hrs/day, 5 days/wk for 12 weeks and utilized EtOH (three-bottle-choice of water, 15%, and 30% EtOH) and Control (water only) treatment groups within each sex. USV recordings were conducted across all experimental phases that included

Baseline, 4-hr EtOH access, 24-hr EtOH access and Abstinence sessions. We then used linear mixed models and binomial logistic regression techniques to explore sex differences in USV total counts and acoustic characteristics (namely mean frequency, duration, bandwidth and power) of 50 – 55 kHz FM and 22 – 28 kHz USVs. Based on our published data and the literature on sex differences, we predicted sex differences in USV profiles and sex-specific effects of alcohol experience on USV counts and acoustic characteristics in male and female HAD-1 rats.

MATERIALS AND METHODS

Subjects

We received 13 male (7 EtOH, 6 control) and 16 female (10 EtOH, 6 control) high-alcohol-drinking rats (HAD-1 generation = 68) from the Alcohol Research Resource Center at the Indiana University School of Medicine at 4 weeks of age. Animals were housed under a reverse light/dark cycle (lights out at 10:00 AM) and were group- and pair-housed in plastic cages (22 x 44 x 20 cm). Animals were handled daily for 4 weeks prior to the start of the experiment in a behavioral testing room separate from the vivarium. Animals were group-housed in wire-topped plastic cages (22 x 44 x 20 cm) until 1 week prior to the start of the experiment when they were single-housed and remained single-housed thereafter, throughout the duration of the experiment. Rats received food and water ad libitum throughout the entire experiment and were weighed 5 days per week just after lights out. The University of Texas at Austin Institutional Animal Care and Use Committee (IACUC) granted prior approval for all experimental procedures.

Procedures

Experimental Sessions

At the beginning of the dark cycle, animals were weighed and then transported from the vivarium to the behavioral testing room. The 4-hr experimental sessions were conducted 5 days per week (in the dark with only red illumination) and commenced for a total of 12 weeks. USV recordings were collected three days per week (first, third and fifth day of each week) from each rat during the 4-hr experimental sessions, including each alternating day of alcohol access during the 24-hr EtOH Access phase. During the first two weeks (Baseline), all rats had access to three sipper tubes filled with water only. For the next phase (4-hr EtOH Access; 4 wks) the EtOH group had access to a 3-bottle choice of water, 15% and 30% EtOH (Bell et al., 2006, 2011) while the Control group had access to three water sipper tubes during the session. For the next phase (24-hr EtOH Access; 4 wks) animals in the EtOH group had 24-hr access to ethanol every other day. During this phase, animals had EtOH access during the entire 4-hr experimental session as in the previous phase (e.g., 4-hr EtOH Access) and then received continued EtOH access in their home cage for the remaining 20-hrs (same 3 bottles as used in the experimental session). For the last experimental phase (Abstinence; 2 wks) all animals had access to three sipper tubes of water during the experimental session. Fluid intake was assessed gravimetrically after each drinking interval.

Apparatus

The experimental chambers were identical to home cages, with the addition of ultrasonic microphones (Avisoft Bioacoustics, Berlin, Germany) affixed to the top center of a sealed Plexiglas cover. Each animal was assigned to the same specific test chamber

each day to control for nonspecific USV emissions induced by novel environments and conspecific odors (Wohr et al., 2008).

USV Recordings

Ultrasonic vocalizations (USVs) were recorded across a range of 10 – 250 kHz using CM16 microphones stored on a PC using an UltraSoundGate interface (Avisoft Bioacoustics, Berlin, Germany) at a sampling rate of 250 kHz with 16-bit resolution. Within the test chamber, approximate distances between the microphone center and the animal's head during test sessions could range from 5 cm to 28.4 cm.

USV Analyses and Algorithm Criterion

Frequency-modulated (FM) 50 – 55 kHz and 22 – 28 kHz USV counts were quantified using the WAAVES algorithm as previously described (Reno et al., 2013). Briefly, the WAAVES algorithm applies a set of conditions to define 50 – 55 kHz FM and 22 – 28 kHz USVs and to filter out noise elements. The WAAVES algorithm defined FM 50 – 55 kHz USVs as sound units occurring within a frequency range of 30 – 120 kHz with a 5-ms minimum duration and variation of 5 kHz or more over the entire USV duration. To determine separation between individual 50 – 55 kHz USVs, the inter-call interval was set at 10-ms or greater. 22 – 28 kHz USV calls were defined by WAAVES as those occurring within the frequency range of 20 – 30 kHz with a minimum duration of 200 ms. To differentiate between successive 22 – 28 kHz USVs and avoid multiple counts of a single, long duration USV, the minimum inter-call interval was set at 100 ms. Once the calls are identified, several measurements of interest are extracted from each USV call and stored for subsequent analysis. The mean frequency, duration, bandwidth, and power for both 50 – 55 kHz FM and 22 – 28 kHz calls were used for statistical analysis (Fig. 1).

Validation Process for WAAVES Automation

Research staff blind to experimental conditions manually analyzed subsets of USV data collected during experimental sessions (thirty 10-min USV files for each of the 50 – 55 and 22 – 28 kHz ranges) by visual and auditory examination of USV spectrograms. The same data sets were then analyzed using the WAAVES program to enable comparisons between WAAVES- and human-derived counts. Final comparisons showed high correspondence for both 50 – 55 kHz FM (R = 0.9606) and 22 – 28 kHz (R = 0.9798) USV subtypes.

Statistical Analyses

USV Counts and Acoustic Characteristics

A standard statistical approach would utilize repeated measures ANOVA to analyze the USV data. In this approach, all calls emitted by a rat are used to calculate an average, and then any potential group differences in these averages are assessed. Thus, this method results in loss of important information pertaining to the inter-individual variability in USV calls for each rat, which, in turn, reduces statistical power. To overcome these problems, we used linear mixed models to examine the effect of sex (e.g. Male vs Female) or treatment (e.g. EtOH vs Control) on total USV counts and the pattern of USV acoustic characteristics (e.g. mean frequency, duration, bandwidth, power).

Linear Mixed Models: We assessed differences in total USV counts and each of the four USV characteristics as a function of sex, treatment, or experimental stage using a linear mixed model (LMM) in R (R Core Team, 2015) using the package "lmerTest" (Kuznetsova et al., 2016). The linear models were generated to assess the effect of sex, treatment, experimental phase, or an interaction of these factors for each of the 4 acoustic characteristics of interest. Whenever a significant effect was observed a new reduced

model was generated by removing the significant factor and compared with the original model using an ANOVA in order to assess the impact of the respective factor on the goodness-of-fit for the model. If a significant interaction was observed, post-hoc analyses were used to further investigate the nature of the interaction. A random slope coefficient was included to protect against potential noise introduced by random day-to-day variation in call parameters for each rat.

Binomial Logistic Regression: While LMM focuses on each acoustic property in isolation, we applied binomial logistic regression (BLR) to assess the combined interactive effect of all four USV characteristics to determine if a linear combination of these data was capable of distinguishing between male and female rats. We have previously used this method to accurately discriminate between male high alcohol-drinking (HAD-1) and low alcohol-drinking (LAD-1) rats solely on the basis of their USV acoustic characteristics (Mittal et al., 2017b). A linear combination of the multivariate data is used to calculate a probability value that represents the maximum separation between the groups. Thus, the BLR model can be used to determine whether USVs, across acoustic characteristics, emitted by male rats differ from those emitted by female rats. Because we were interested in examining the ability of these acoustic characteristics to distinguish between male and female rats, we assessed all USVs emitted by each group (e.g. EtOH and Control) at each stage of the experiment (Baseline, 4-hr and 24-hr EtOH Access, Abstinence).

Since the data are used in building the model, it is possible that the best fitting model would be specific to the data used and may not necessarily generalize to the population as a whole. To address this issue and ensure the generalizability of the model, we split the data into a training and testing subset; where one half of the animals are used

to train the model and the remaining half are used to test it. When dividing the groups into training and testing subsets, it is possible that certain combinations of animals within each subset may be more (or less) representative of the entire dataset and, in turn, bias the ability of the model to accurately separate the groups. Thus, in order to produce an accurate assessment, we repeated the binomial logistic regression 10,000 times, each time randomly selecting half of the data as our training set and using the remaining half to test the model. We then computed the percent of animals correctly assigned to their group[2] for each of the 10,000 iterations. The resulting distribution allows us to estimate the average percent correct and standard error for each iteration, thus allowing us to compute 95% confidence intervals around the mean percent correct for the 10,000 trials. If the model performs no better than chance alone, we would expect 50% of the animals to be correctly categorized. Therefore, if the 95% confidence interval around the average percent correct includes 50% we cannot conclude that the model is performing better than chance at an alpha level of 0.05.

Daily EtOH and water intake

Mixed-design ANOVAs were used to compare weekly EtOH intake between the Male and Female EtOH Groups across the 12 weeks of the study. The same test was used to compare fluid intake between the two EtOH and Control Groups.

[2] To compute the percentage of animals correctly assigned to their groups by the BLR we first computed the average logistic regression values across all USVs emitted by each animal. Next, we combined the average USV logistic regression values for each animal to compute the group averages for male and female HAD-1 rats. We then calculated the midpoint between these two means and used this midpoint as the decision boundary for separation. The animals were then classified as male or female based on the side of the decision boundary on which their logistic regression values clustered.

RESULTS

Total USV Counts

50 – 55 kHz FM USVs

We began by examining total call counts for the 50 – 55 kHz FM USVs emitted by male and female HAD-1 rats during the four stages of the experiment. First, we tested whether there was a three-way interaction between sex (i.e. Male vs. Female), treatment (i.e. EtOH vs. Control) and time (i.e. Baseline vs. 4-hr EtOH vs. 24-hr EtOH vs. Abstinence). Although we did not see a Sex*Treatment*Time interaction in the number of 50 – 55 kHz FM USVs emitted, we did find a significant Sex*Time interaction in total call counts for the EtOH, but not the Control group. There was a significant interaction effect on the quadratic ($p < 0.05$, $t_{587.90} = 2.330$; Fig. 2a), but not the linear or the cubic terms of the model. Removal of this interaction resulted in a significant reduction in the model's goodness-of-fit ($p < 0.05$, $\chi^2 = 7.8761$). Post hoc analysis showed that female HAD-1 rats emitted significantly more 50 – 55 kHz FM USVs than male HAD-1 rats during the 24-hr EtOH access period in the EtOH treatment, but not the control group ($p < 0.01$, $t_{44.69} = -2.799$). On the other hand, female HAD-1 rats emitted significantly more 50 – 55 kHz FM USV than male HAD-1 rats during the Abstinence period in the control, but not the EtOH group ($p < 0.05$, $t_{14.276} = -2.843$). A similar trend was seen in the 24-hr EtOH access phase for the control group ($p = 0.0794$, $t_{15.30} = -1.879$), however, this was not statistically significant.

22 – 28 kHz USVs:

We did not observe any Sex*Treatment*Time interaction for the total number of 22 – 28 kHz USVs emitted (Fig. 2b). Independent assessment of the two treatment groups

also did not reveal any Sex*Time interaction nor any main effect of sex on the total call counts of the 22 – 28 kHz USVs.

Binomial Logistic Regression

After assessing the differences between male and female rats on total emitted calls, we sought to examine whether it was possible to discriminate between these groups by using a combination of the acoustic characteristics (i.e. mean frequency, duration, bandwidth, and power) of their USV calls. One way to achieve this is to use binomial logistic regression, a statistical and machine-learning method used to separate two or more classes of objects (e.g. Male vs. Female) based on a linear combination of explanatory variables. To achieve this aim, we split our data into "testing and "training" subsets and used the bootstrapping approach described in the statistical methods above. Once we were confident that the BLR model could accurately classify the two sexes we generated a new equation using the entire data set in order to calculate the coefficients associated with each acoustic characteristic. Since, the USV count data suggest that the potential sex differences in USV acoustic characteristics can vary based on the treatment and the stage of the experiment, different logistic regression models were constructed for Control and EtOH groups at each phase of the experiment (Baseline, 4-hr and 24-hr EtOH Access, Abstinence). The mean accuracy and the 95% confidence interval for the 10,000 bootstrapped iterations for each stage are illustrated in Fig. 3.

50 – 55 kHz FM USVs:

A linear combination of 50 – 55 kHz FM USV characteristics could be used to design a logistic regression model capable of discriminating between male and female HAD-1 rats with reasonable accuracy (Fig. 3a). Moreover, the ability of this model to distinguish between male and female HAD-1 rats was associated with EtOH access. Male

and female Controls could be consistently classified with a mean accuracy of approximately 80% at all stages of the experiment. However, the classification accuracy of EtOH rats was better than Controls in the presence of EtOH (e.g., during 4-Hr [EtOH = 82.16% vs. Control = 70.07%] and 24-Hr EtOH Access phases [EtOH = 93.86% vs. Control = 85.36%]), but worse in the absence of EtOH (e.g., during Baseline [EtOH = 48.58% vs. Control = 82.03%] and Abstinence [EtOH = 72.75% vs. Control = 86.39%]). The logistic regression model coefficients are listed in Table 1 below.

22 – 28 kHz USVs:

Similar to the 50 – 55 kHz FM USV characteristics data, binomial logistic regression models were able to accurately discriminate between male and female HAD-1 rats based on the 22 – 28 kHz USV acoustic characteristics (Fig. 3b). However, unlike the 50 – 55 kHz FM models, we did not see any clear differences between the control [Baseline = 66.36%; 4-Hr EtOH = 88.48%; 24-Hr EtOH = 88.46%; Abstinence = 74.69%] or ethanol [Baseline = 72.05%; 4-Hr EtOH = 69.29%; 24-Hr EtOH = 88.45%; Abstinence = 63.96%] treated rats on the discrimination accuracy of the models based on 22 – 28 kHz USV acoustic characteristics. The logistic regression model coefficients are shown in Table 2 below.

The magnitude of the binomial logistic regression coefficients is weighted such that the acoustic characteristics with the largest coefficients contribute the most to the sex differences observed in the model. For instance, the mean frequency for both 50 – 55 kHz FM and 22 – 28 kHz USVs has the largest coefficients across multiple stages of the experiment, suggesting that mean frequency of USVs may differ significantly between male and female HAD-1 rats. In order to directly explore these differences, we next used linear mixed models to assess the effect of sex, treatment and experiment stage, as well

as, an interaction between these factors on the acoustic characteristics of both 50 – 55 kHz FM and 22 – 28 kHz USVs.

50 – 55 kHz FM USV Acoustic Characteristics

Mean Frequency:

We observed a significant Sex*Treatment*Time interaction for the mean frequency of 50 – 55 kHz FM USVs (Fig. 4a). Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model ($p < 0.0001$, $\chi^2 = 29.636$). Independent assessment of the two treatment groups revealed a significant Sex*Time interaction for EtOH ($p < 0.0001$, $\chi^2 = 496.21$) and Control ($p < 0.0001$, $\chi^2 = 185.67$) groups. Post hoc analyses revealed that males in the EtOH group emitted 50 – 55 kHz FM USVs with a significantly higher mean frequency than EtOH females during 24-hour EtOH Access ($p < 0.0001$, $t_{14.623} = 7.166$) and Abstinence ($p < 0.05$, $t_{10.959} = 2.598$). Male Controls also emitted 50 – 55 kHz USVs of higher mean frequency than their female counterparts during 24-hour EtOH Access ($p < 0.05$, $t_{10.067} = 2.788$), but not during Abstinence.

Duration:

We did not observe any Sex*Treatment*Time interaction for the duration (Fig. 4b) of 50 – 55 kHz FM USVs. However, independent assessment of the two treatment groups revealed a significant Sex*Time interaction for the EtOH ($p < 0.05$, $\chi^2 = 9.7696$) and a main effect of sex for the Control group ($p < 0.05$, $\chi^2 = 5.5429$). Post hoc analyses did not reveal any further sex differences in the EtOH group during any of the four experiment stages. However, male Controls emitted significantly longer 50 – 55 kHz FM calls during the 4-hour Access phase ($p < 0.05$, $t_{8.726} = 2.496$) compared to their female counterparts.

Bandwidth:

We did not observe any Sex*Treatment*Time interaction for the bandwidth (Fig. 4c) of 50 – 55 kHz FM USVs. Independent assessment of the two treatment groups also did not reveal any Sex*Time interaction nor any main effect of sex on the bandwidth of 50 – 55 kHz FM USVs.

Power (dB):

There was a significant Sex*Treatment*Time interaction for the power of 50 – 55 kHz FM USVs (Fig. 4d). Removal of this effect resulted in a significant reduction in the goodness-of-fit of the model ($p < 0.01$, $\chi^2 = 15.271$). Independent assessment of the two treatment groups revealed a significant Sex*Time interaction for both EtOH ($p < 0.0001$, $\chi^2 = 73.445$) and Control ($p < 0.0001$, $\chi^2 = 59.973$) groups. Post hoc analyses revealed that 50 – 55 kHz USVs emitted by EtOH males were louder than their female counterparts during 4-hr ($p < 0.05$, $t_{14.226} = -2.175$) and the 24-hr EtOH access periods ($p < 0.05$, $t_{14.785} = -2.918$). No further sex differences were observed in the Control group at any of the four experiment stages.

22 – 28 kHz USV Acoustic Characteristics

Mean Frequency:

There was a significant Sex*Treatment*Time interaction for 22 – 28 kHz USV mean frequency (Fig. 5a). Removal of this effect resulted in a significant reduction in the goodness-of-fit of the model ($p < 0.0001$, $\chi^2 = 159.09$). Independent assessment of the two treatment groups revealed a significant Sex*Time interaction for both EtOH ($p < 0.0001$, $\chi^2 = 222.77$) and Control ($p < 0.0001$, $\chi^2 = 131.48$) groups. Post hoc analyses revealed that mean frequency of 22 – 28 kHz USVs emitted by EtOH males were significantly lower than their female counterparts during 4-hr ($p < 0.05$, $t_{13.030} = -2.17$) and 24-hr EtOH

Access periods ($p < 0.001$, $t_{15.194} = -4.714$).While the mean frequency USVs in the 22 – 28 kHz range was lower in Control male rats compared to their female counterparts during weeks 7-10 (e.g., "24-hr access" phase; $p < 0.01$, $t_{7.911} = -4.745$), and weeks 11-12 (e.g., "Abstinence"; $p < 0.05$, $t_{8.116} = -2.524$).

Duration:

There was a significant Sex*Treatment*Time interaction for 22 – 28 kHz USV duration (Fig. 5b). Removal of this effect resulted in a significant reduction in the goodness-of-fit of the model ($p < 0.001$, $\chi^2 = 17.944$). Independent assessment of the two treatment groups revealed a significant Sex*Time interaction for both EtOH ($p < 0.01$, $\chi^2 = 14.981$) and Control ($p < 0.0001$, $\chi^2 = 127.88$) groups. Post hoc analyses showed that EtOH males emitted longer 22 – 28 kHz USVs than their female counterparts during Baseline ($p < 0.01$, $t_{10.06} = 3.787$). No further sex differences were observed in the EtOH group during 4-hr, 24-hr EtOH access or Abstinence phases. No sex differences in 22 – 28 kHz USV duration were observed in Controls.

Bandwidth:

There was a significant Sex*Treatment*Time interaction for the bandwidth of 22 – 28 kHz USVs (Fig. 5c). Removal of this effect resulted in a significant reduction in the goodness-of-fit of the model ($p < 0.001$, $\chi^2 = 17.596$). Independent assessment of the two treatment groups revealed a significant Sex*Time interaction for both EtOH ($p < 0.0001$, $\chi^2 = 120.79$) and Control ($p < 0.0001$, $\chi^2 = 380.58$) groups. Post hoc analyses showed that male rats in both EtOH ($p < 0.05$, $t_{11.326} = -2.463$) and Control ($p < 0.05$, $t_{11.541} = -2.631$) groups emitted narrower bandwidth 22 – 28 kHz USVs compared to their female counterparts during Abstinence (e.g., weeks 11-12). Post hoc analyses did not reveal any

further sex differences in EtOH or Control groups during Baseline, 4-hr or 24-hr EtOH Access.

Power (dB):

There was a significant Sex*Treatment*Time interaction for 22 – 28 kHz USVs power (Fig. 5d). Removal of this effect resulted in a significant reduction in the goodness-of-fit of the model ($p < 0.001$, $\chi^2 = 19.527$). Independent assessment of the two treatment groups revealed a significant Sex*Time interaction for EtOH ($p < 0.0001$, $\chi^2 = 200.73$) and Controls ($p < 0.0001$, $\chi^2 = 405.67$). Post hoc analyses did not reveal any further sex differences in EtOH or Control groups during the Baseline, 4-hr and 24-hr EtOH Access or Abstinence phases.

EtOH Intake during USV Recording Sessions (4-hr Intake Levels)

During the 4-hr drinking phase, three bottles (15% EtOH, 30% EtOH and water) were placed in the cages at the beginning of the USV recording session. At the end of the 4-hour recording session the bottles were removed and weighed to measure alcohol consumption. During the 24-hr drinking phases, the bottles were measured at the end of the 4-hr recording session, and placed back on the cages for an additional 20 hrs. Analyses of 8 wks of EtOH drinking data include both the 4-hr and 24-hr EtOH Access phases.

Both male and female HAD-1 rats gradually acquired EtOH drinking to pharmacologically relevant levels. A comparison of the EtOH dose (g/kg) during each week of drinking between male and female HAD-1 rats was performed using a 2-group x 8-week mixed design ANOVA. Females drank significantly more than the males ($F_{1,15} =$

12.52, $p < 0.01$; Fig. 6a), and EtOH consumption increased over time ($F_{7,105} = 30.30$, $p < 0.0001$).

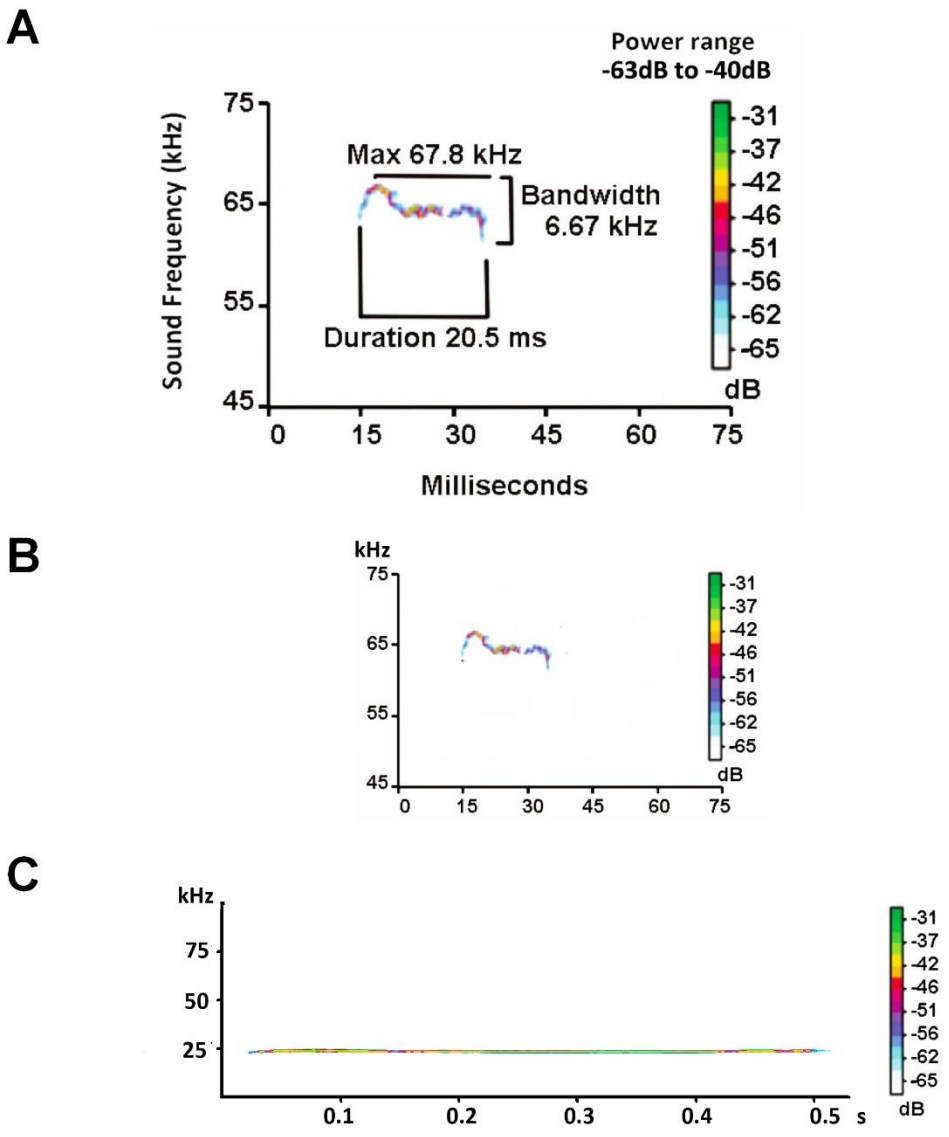
Total EtOH Intake during 24-hr Drinking Phase

A 2-group x 4-week mixed design ANOVA revealed that the females also drank significantly more than males during the 24-hour drinking sessions ($F_{1,15} = 13.93$, $p < 0.01$; Fig. 6b), and EtOH consumption increased over the 4-week period ($F_{3,45} = 12.29$, $p < 0.0001$).

Total Fluid Intake During All Phases

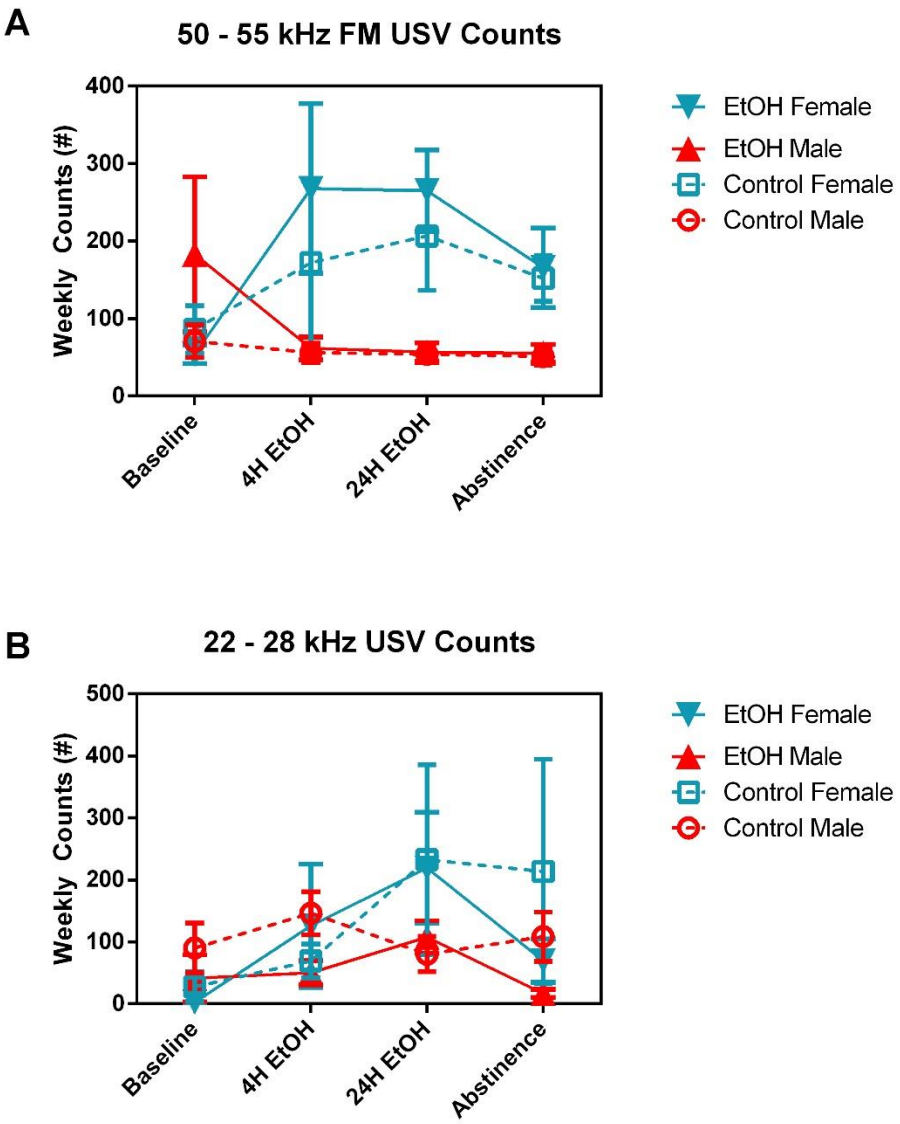
Three-way ANOVA applied to the total fluid consumption over the 12-week experiment did not reveal any interaction or main effect of sex or treatment over time (Fig. 6c). Though there was a significant increase in total fluid consumption over time for all groups ($F_{11,275} = 18.48$, $p < 0.0001$).

Illustration 1: Schematic of representative spectra for different USV subtypes.



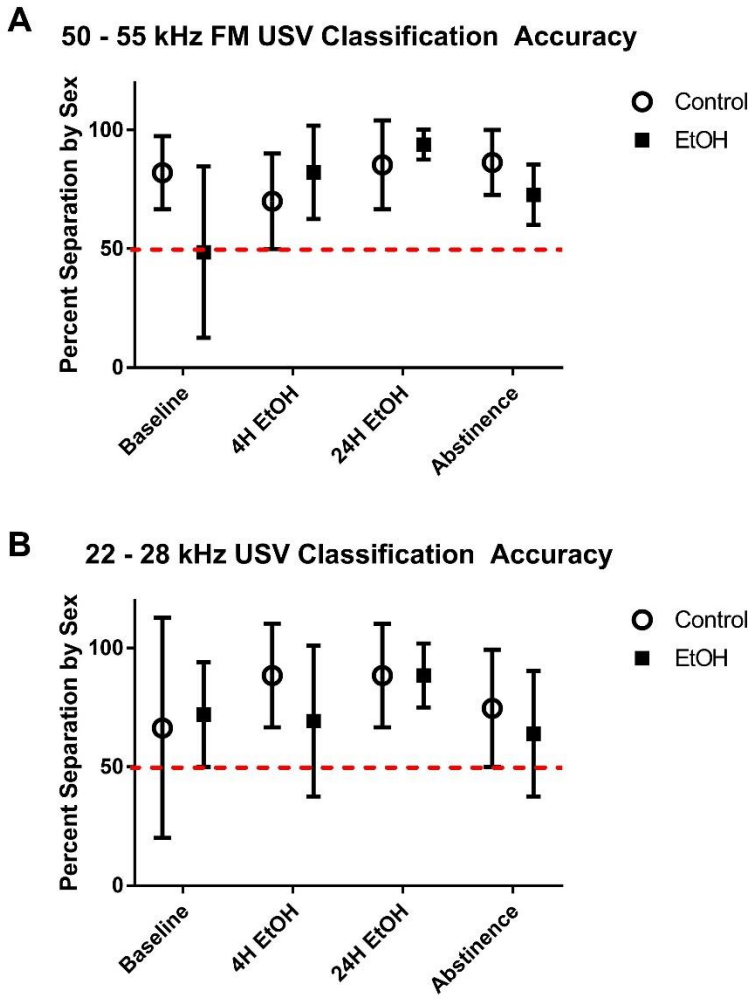
A) Exemplary sonogram of positive-affect (50 – 55 kHz FM) USV with explanation of acoustic characteristics. Power (loudness) is measured in decibels (dB). B) Example of a frequency modulated (FM) 50 – 55 kHz USV C) Example of a 22 – 28 kHz USV.

Figure 1: Sex differences in total USV counts between male and female HAD-1 rats.



Linear mixed models were used to assess the effect of sex, treatment and time on total USV emissions of male and female HAD-1 rats. A) Female rats emitted more 50 – 55 kHz calls than male rats in both Control and EtOH groups, but the effect was only significant for the EtOH group ($p < 0.05$). B) There were no differences in 22 – 28 kHz USV emissions between male and female rats. Mean ± sem of average weekly call counts are reported.

Figure 2: Binomial logistic regression analysis: 50 – 55 kHz FM and 22 – 28 kHz USV acoustic characteristics



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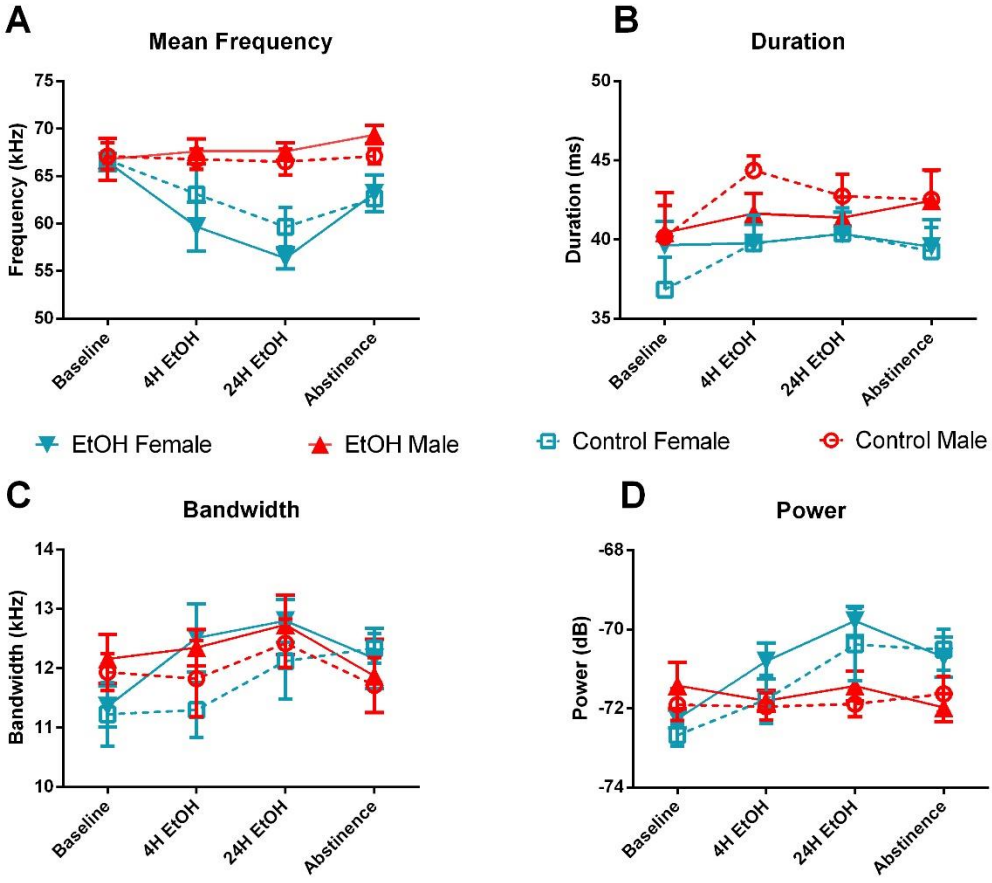
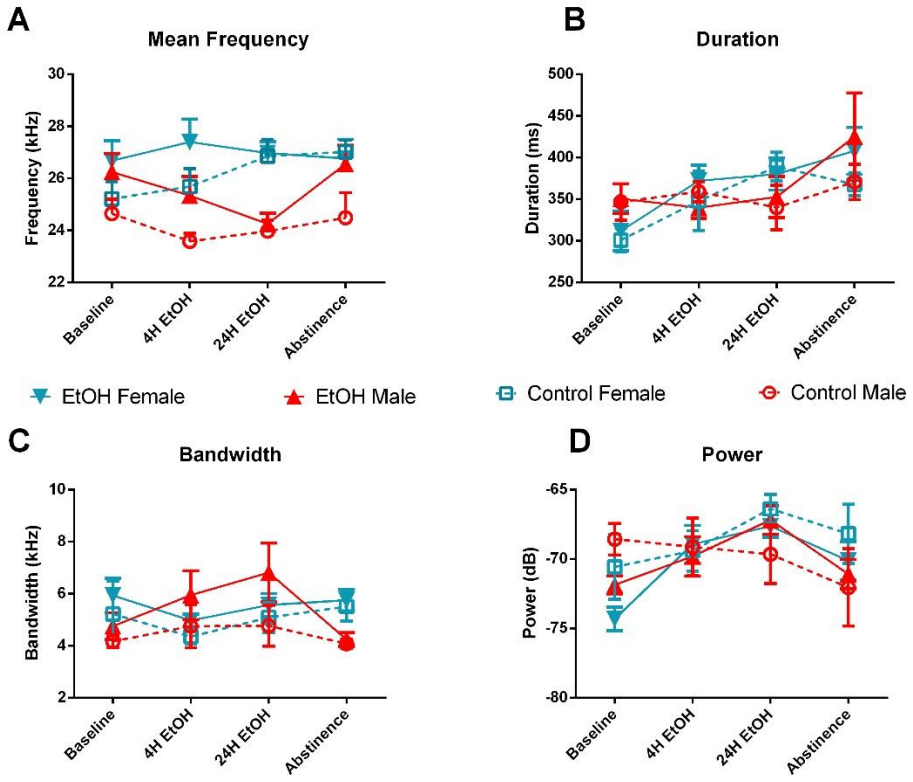
Binomial logistic regression (BLR) was used to assess whether a combination of USV acoustic characteristics (i.e. mean frequency, duration, bandwidth, and power) could be used to discriminate between male and female HAD-1 rats. Using a bootstrapping approach BLR was simulated 10,000 times and the mean accuracy and 95% confidence interval for the discrimination accuracy at each stage are illustrated. The red line represents 50% accuracy, thus, if the mean and 95% confidence fall above the red line, the ability of the model to classify HAD-1 rats based on sex is considered to be better than random chance. A) For 50 – 55 kHz FM USV characteristics, the classification accuracy of EtOH rats was better than Controls in the presence of EtOH (e.g., during 4-Hr [EtOH = 82.16% vs. Control = 70.07%] and 24-Hr [EtOH = 93.86% vs. Control = 85.36%] EtOH Access phases), but worse in the absence of EtOH (e.g., during Baseline [EtOH = 48.58% vs. Control = 82.03%] and Abstinence [EtOH = 72.75% vs. Control = 86.39%]). B) There were no clear trends in the classification accuracy of the models based on 22 – 28 kHz USV characteristics was similar for Control [Baseline = 66.36%; 4-Hr EtOH = 88.48%; 24-Hr EtOH = 88.46%; Abstinence = 74.69%] and EtOH [Baseline = 72.05%; 4-Hr EtOH = 69.29%; 24-Hr EtOH = 88.45%; Abstinence = 63.96%] groups. Mean ± 95% confidence interval for the model classification accuracy are reported.

Figure 3: 50 – 55 kHz FM USV acoustic characteristics of male and female HAD-1 rats.



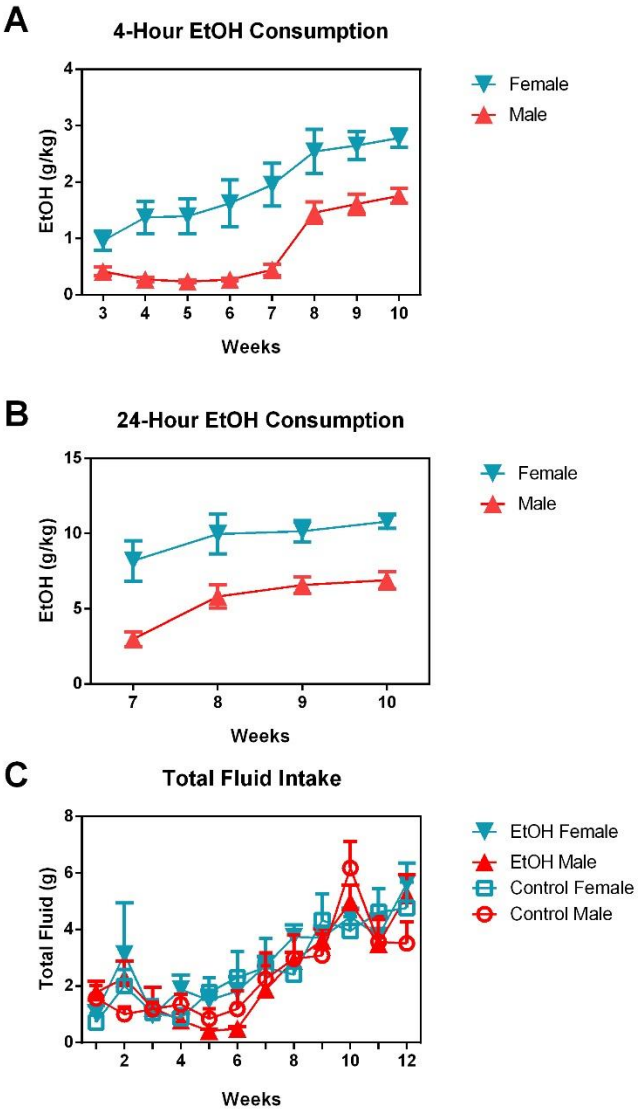
Linear mixed models were used to assess the effect of sex and treatment on the acoustic characteristics of spontaneously emitted 50 – 55 kHz frequency modulated (FM) USVs. A) The mean frequency of 50 – 55 kHz FM USVs was comparable between males and females at Baseline, but decreased over time in EtOH and Control female rats. Males in the EtOH group emitted calls with a higher mean frequency than females during both 24-hour EtOH Access ($p < 0.0001$) and Abstinence ($p < 0.05$), while males in the Control group only emitted calls with a higher mean frequency during 24-hour Access stage ($p < 0.05$), but not during the Abstinence stage. B) The duration of calls emitted by males was higher than those of females in both the EtOH and Control groups. C) There were no sex differences on the bandwidth of calls emitted by male and female HAD-1 rats. D) There was an increase in the power of calls emitted by female, but not male HAD-1 rats in both the EtOH and Control groups. Calls emitted by males in the EtOH group were louder than their female counterparts during 4-hr ($p < 0.05$) and the 24-hr EtOH access periods ($p < 0.05$). Mean ± sem for each USV acoustic characteristic are reported.

Figure 4: 22 – 28 kHz USV acoustic characteristics of male and female HAD-1 rats.



Linear mixed models were used to assess the effect of sex and treatment on the acoustic characteristics of spontaneously emitted 22 – 28 kHz USVs. A) The mean frequency of the 22 – 28 kHz calls emitted by female rats showed a slight increase over time, while the mean frequency of the calls emitted by male rats decreased over the course of the experiment. Males in the EtOH group made calls with significantly lower mean frequencies than their female counterparts during 4-hr ($p < 0.05$) and 24-hr EtOH Access periods ($p < 0.001$), while those in the control group made calls with lower frequencies than females during the 24-hr EtOH Access periods ($p < 0.01$) and Abstinence periods ($p < 0.05$). B) At Baseline, USVs were of shorter duration in EtOH treated females compared to males ($p < 0.01$). 22 – 28 kHz calls emitted by both EtOH and Control females, as well as, EtOH males increased over time. The duration of these calls for Control males did not significantly change over time. C) The bandwidth of 22 – 28 kHz USVs was significantly lower for males compared to females during Abstinence in both Control ($p < 0.05$) and EtOH ($p < 0.05$) groups. D) For Controls, the power of 22 – 28 kHz USVs emitted by males decreased over time, while increasing over time in females. In the EtOH group, males made louder 22 – 28 kHz calls than females at Baseline, but not thereafter. Mean ± sem for each USV acoustic characteristic are reported.

Figure 5: Sex differences in EtOH, but not total fluid consumption between male and female HAD-1 rats.



Mixed ANOVA was used to assess the effect of sex on EtOH and total fluid consumption of male and female HAD-1 rats. Female HAD-1 rats consumed significantly more alcohol than the male rats during both A) the 4-hour ($p < 0.01$) and B) the 24-hour ($p < 0.01$) EtOH access stages. C) No sex differences were observed in total fluid consumption during any stage of the experiment. Mean ± sem for average daily fluid and ethanol consumption are reported.

Table 1: Binomial logistic regression coefficients for 50 – 55 kHz FM USVs.

BINOMIAL LOGISTIC REGRESSION COEFFICIENTS FOR 50 – 55 KHZ FM USVS.

Experiment Stage	β_0	$\beta_{Mean\ Frequency}$	$\beta_{Duration}$	$\beta_{Bandwidth}$	β_{Power}
Baseline Control	-0.19596	0.02337	0.14146	0.02697	0.13529
Baseline EtOH	0.95054	-0.40683	-0.16556	0.02573	0.41804
4-Hr Control	-1.3428	0.9293	0.1488	0.1959	-0.2213
4-Hr EtOH	-2.32595	1.24327	0.01716	0.15788	-0.02601
24-Hr Control	-1.5919	0.7952	0.1588	0.2176	-0.4376
24-Hr EtOH	-2.28276	1.02120	0.06452	0.18175	-0.14114
Abstinence Control	-1.17340	0.48963	0.16468	0.03189	-0.25608
Abstinence EtOH	-1.68204	0.79152	0.17368	0.08333	-0.28652

Note. The coefficients represent the β values associated with the intercept (β_0) and each acoustic characteristic used to calculate the log odds ratio for the 50 – 55 kHz frequency modulated (FM) calls. The magnitude of these coefficients represents the contribution of the respective acoustic characteristic to the total separation achieved by the logistic regression model.

Table 2: Binomial logistic regression coefficients for 22 – 28 kHz USVs.

BINOMIAL LOGISTIC REGRESSION COEFFICIENTS FOR 22 – 28 KHZ USVS.

Experiment Stage	β_0	$\beta_{Mean\ Frequency}$	$\beta_{Duration}$	$\beta_{Bandwidth}$	β_{Power}
Baseline Control	1.22605	-0.27036	0.29994	-0.07374	0.24169
Baseline EtOH	3.2879	-0.7979	-0.6973	-0.4549	0.7429
4-Hr Control	0.9016	-1.3112	0.1433	0.4671	0.3744
4-Hr EtOH	-2.4384	-2.0333	-0.3986	0.7467	-0.6140
24-Hr Control	-2.02783	-2.26365	0.24595	-0.08992	-0.71993
24-Hr EtOH	-2.0128	-2.1887	-0.2234	0.3300	0.6538
Abstinence Control	-1.5135	-2.3598	0.4585	-1.0580	-0.9102
Abstinence EtOH	-2.1589	0.1378	0.3251	-0.4974	-0.8526

Note. The coefficients represent the β values associated with the intercept (β_0) and each acoustic characteristic used to calculate the log odds ratio for the 22 – 28 kHz calls. The magnitude of these coefficients represents the contribution of the respective acoustic characteristic to the total separation achieved by the logistic regression model.

DISCUSSION

This study examined the effects of sex and alcohol experience on USV emissions as a means to examine relationships between alcohol consumption and emotional status in male and female HAD-1 rats. 22 – 28 kHz and 50 – 55 kHz FM USV data collected over 12-weeks of study included USVs counts and acoustic characteristics emitted before, during and after alcohol exposure. From these data, a number of interesting results emerged that were in agreement with our predictions of sex differences in USV profiles and sex-specific effects of alcohol experience in male and female HAD-1 rats.

Consistent with, and extending previous observations (Thakore et al., 2016), we found that both male and female HAD-1 rats emit a large number of spontaneous 22 – 28 kHz USVs as well as 50 – 55 kHz FM USVs. In addition, females emitted more 50 – 55 kHz FM USVs than males across all phases of the experiment, though the difference was statistically significant only during the 24-hr EtOH Access phase (e.g., weeks 7-10). Binomial logistic regression analyses showed that USV acoustic characteristics accurately discriminate between male and female HAD-1 rats. In addition, the separation accuracy for 50 – 55 kHz FM calls was modulated by the presence or absence of EtOH, whereas the separation accuracy for the 22 – 28 kHz calls was unaffected by treatment. Finally, we used linear mixed models to directly examine sex differences in the mean frequency, duration, bandwidth and power of 50 – 55 kHz FM and 22 – 28 kHz USVs and found significant sex differences that were differentially modulated by EtOH exposure. This study provides the first direct evidence for the use of USVs as reliable markers of sex differences in HAD-1 rats and demonstrates the sensitivity of USV acoustic parameter analyses for detecting alcohol and alcohol experience-induced alterations.

Ultrasonic vocalizations are a means of social communication in rodents that signal biologically relevant affective responses (Knutson et al., 2002; Brudzynski, 2005, 2013; Wöhr and Schwarting, 2013; Wöhr et al., 2015). Since USVs are known to reflect real-time activity in dopaminergic and cholinergic neurotransmitter systems (Brudzynski, 2001; Bihari et al., 2003; Burgdorf et al., 2007; Ciucci et al., 2008; Kurejova et al., 2010; Brudzynski et al., 2011; Wright et al., 2013; Roccaro-Waldmeyer et al., 2016; Williams and Undieh, 2016), USV counts have served as useful assessments of emotional responses during drug use and withdrawal (Ahrens et al., 2009; Maier et al., 2012; Berger et al., 2013). However, high inter-individual variability in USV counts makes it difficult to obtain sufficient statistical power to assess group differences. In addition, the time-consuming aspect of manual USV analysis is another impediment to wide use of USVs in research. However, the recent the introduction of automated USV analysis tools such as the WAAVES program (Reno et al., 2013; Reno and Duvauchelle, 2014), and template matching approaches (Barker et al., 2014; Barker and Johnson, 2017) demonstrate that automated analyses techniques can be used for accurate and timely analyses of USV data.

This study highlights the importance of statistical assays best equipped to maximize the information extracted from high-dimensional datasets such as USV data (Mittal et al., 2017b; Reno et al., 2017). Traditional statistical analyses require the aggregation of data from thousands of USV calls into averages for each individual subject, substantially reducing statistical power in the process and severely hampering the ability to draw inferences with sufficient confidence. Here we show that linear mixed models allow the data from all 22 – 28 kHz (total: 38,481) and 50 – 55 kHz FM (total: 48,453) USV calls to be used in the analysis and accurately assess three-way interactions with a high degree of confidence. Moreover, we also show here that the use of machine learning classification algorithms such as binomial logistic regression allows us to use a

combination of multivariate USV acoustic characteristic data to discriminate between different experimental groups (i.e. male vs. female HAD-1 rats). In conjunction with high-throughput automated USV analyses, powerful statistical models such as LMMs and BLRs reveal the sensitivity of USVs in distinguishing between populations that vary in alcohol consumption levels.

In the present study, females consumed more alcohol than males during both the 4-hr and 24-hr access periods. This finding is in line with the literature on sex differences in alcohol consumption in rodents (Blanchard et al., 1993; Almeida et al., 1998; Juárez and Barrios de Tomasi, 1999; Nieto and Kosten, 2017). However 4-hr EtOH consumption levels for the male HAD-1 rats reported here fall well below our previous findings from 3-hrs of EtOH access during DID sessions (Thakore et al., 2016). One possible explanation points to procedural differences between EtOH drinking sessions in the current study and the DID drinking sessions from our previous report. For instance, our DID drinking sessions consisted of three 1-hr intervals interspersed with 2-hr water-only intervals, while the current drinking procedure consisted of a 4-hr uninterrupted EtOH access interval. Practically speaking, the process of changing out sipper tubes multiple times in each DID session is the equivalent of presenting multiple cues of alcohol availability to the rats. In contrast, during 4-hr EtOH Access, aside from the start of the session, no additional disruptions are associated with alcohol access. Since drinking conditions were the same for male and female rats in the current study, the 4-hr EtOH Access procedure could differentially favor consumption levels in the more attentive sex within the selectively-bred HAD-1 rat line. Though the literature for HAD-1 rats is lacking in this area, we have preliminary behavioral data from object recognition tests (ORT) (Mittal et al., 2017a) showing poor performance by HAD-1 males in an attentional task and enhanced cognitive reactivity in HAD-1 females compared to their

male counterparts. Whether these behavioral tests hold relevance under the conditions of EtOH access discussed here is yet to be determined. However, it is worth considering the potential influence of sex-specific factors on procedural methodologies such as free access to alcohol administration.

By focusing on USV acoustic characteristics and advanced statistical analyses, the current study revealed unique and robust differences in USVs between alcohol-naïve male and female HAD-1 rats. Similarly, significant sex differences were also observed in subsequent alcohol consumption levels. These findings are in agreement with a recent study in our laboratory showing that USV acoustic characteristics can be used to develop machine learning models capable of discriminating between pairs of selectively-bred high and low alcohol-consuming male rats (e.g., P vs. NP and HAD-1 vs. LAD-1 rat lines) (Mittal et al., 2017b; Reno et al., 2017). Although the specific nature of USV acoustic characteristics are not yet well understood, the present study reinforces the association between 22 – 28 and 50 – 55 kHz FM USV acoustic parameters (e.g., mean frequency, duration, bandwidth and power) and differences in drinking levels between male and female HAD-1 rats. Therefore, it is possible that sex differences in the USV acoustic characteristics observed here may reflect differences in underlying neural transmission pathways which drive both USV emissions and alcohol consumption in male and female HAD-1 rats.

Chapter 3: Sex differences in alcohol consumption, object recognition and rearing in male and female high alcohol-drinking (HAD-1) rats

ABSTRACT

Excessive alcohol consumption has a negative impact on social and emotional well-being and the ability to think and reason. Sex differences in alcohol consumption patterns are observed in humans, primates and rodents alike, therefore it is important to identify the neural substrates that might underlie these sex-dependent phenotypes. Rodent models such as selectively bred high alcohol-drinking rat lines, such as the "HAD-1" lines have been successful in furthering our understanding of the biological underpinnings that drive alcohol consumption. The present work examined sex differences in alcohol consumption, object recognition and rearing in male and female HAD-1 rats. Naïve male and female HAD-1 rats were tested in an object recognition test (ORT), followed by 5 wks of 24-hr alcohol consumption sessions. We found that during the initial training trial of ORT female HAD-1 rats spent significantly more time in the section containing the objects than male HAD-1 rats, they actively investigated the objects more and made significantly more rears than males. During the subsequent testing trial, females showed a marginally significant increase in time spent in the section containing the objects compared to males, but object investigation time and number of rears did not statistically differ between females and males. Female HAD-1 rats also consumed significantly more alcohol than their male counterparts. Moreover, across all animals there was a significant correlation between exploratory behavior and alcohol consumption levels. These results indicate significant sex differences in cognitive and/or emotional reactivity and alcohol consumption in female and male HAD-1 rats.

INTRODUCTION

Alcohol use disorder is a widely prevalent and debilitating mental disorder with significant negative medical consequences and a vast societal socioeconomic cost. In the US, the estimated cost associated with excessive alcohol consumption in 2010 was $ 249.0 billion (Sacks et al., 2015). Therefore, it is important to identify the neurobiological correlates that underlie excessive and hazardous alcohol consumption. Although a variety of genetic and environmental factors associated with alcohol abuse and dependence have been identified (Enoch, 2014; Reilly et al., 2017), the interaction of these factors with gender and sex-associated differences in the brain remains largely unexplored (Becker and Koob, 2016; Becker et al., 2017).

Historically men consume more alcohol than women, however studies show that this gap has narrowed in recent decades (Substance Abuse and Mental Health Services Administration and Administration, 2014; White et al., 2015). While non-human primates show similar sex differences in alcohol consumption (Vivian et al., 2001), female rodents drink more than their male counterparts (Juárez and Barrios de Tomasi, 1999). In order to characterize the neurological underpinnings of differential alcohol consumption in rodents, we sought to identify non-invasive behavioral markers that correspond with sex differences in alcohol consumption between male and female high alcohol-drinking (HAD-1) rats. Recent studies have shown that forced novelty seeking in a hole-board apparatus (Manzo et al., 2014), novelty-induced locomotor activity (Barson et al., 2013), and vertical exploration (rearing) in a novel activity chamber (Pandey et al., 2017) to be reliable predictors of excessive alcohol consumption in rodents.

In this study we used a one-trial novel-object recognition task to investigate whether differences in novelty seeking, object recognition and rearing behaviors existed between male and female HAD-1 rats. Although the object-recognition task is primarily

considered a test of working memory (Ennaceur and Delacour, 1988; Dere et al., 2007), the initial exploration of the test chamber and investigation of the object can serve as reliable indices of novelty-seeking. We hypothesized that, in line with higher ethanol consumption levels, we would see increased exploratory activity and novelty-seeking behaviors in female HAD-1 rats.

MATERIALS AND METHODS

Subjects

We received 8 male and 8 female high-alcohol-drinking rats (HAD-1 generation = 68) from the Alcohol Research Resource Center at the Indiana University School of Medicine at 4 weeks of age. Animals were housed under a reverse light/dark cycle (lights out at 10:00 AM) and were group- and pair-housed in plastic cages (22 x 44 x 20 cm). Animals were handled daily for 4 weeks prior to the start of the experiment in a behavioral testing room separate from the vivarium. Animals were group-housed in wire-topped plastic cages (22 x 44 x 20 cm) until 1 week prior to the start of the experiment when they were single-housed and remained single-housed thereafter, throughout the duration of the experiment. Rats received food and water ad libitum throughout the entire experiment. The University of Texas at Austin Institutional Animal Care and Use Committee (IACUC) granted prior approval for all experimental procedures.

Novel Object Recognition Test

Two days before the ORT, animals were habituated to the test chambers for 20 minutes per day. No objects were present in the chamber during the habituation periods. On the third day, the ORT was performed in two phases, a Training Phase and a Testing

Phase. For the Training Phase, each rat was placed in an open field for 600 seconds. The floor of the arena was divided into four quadrants labeled 1-4 and two identical objects were placed in quadrant 1. Rats were videotaped while exploring the open field for 10 minutes. The rat was then removed, placed back in the home cage for one hour and then entered the Test Phase. Here, all procedures were identical except that one of the objects was replaced with a novel object. Time in each section, time in contact with each object, and total number of rears in each section were recorded for both training and testing phases. Two-way ANOVA was used to assess the effect of sex on the recorded behaviors.

Ethanol availability sessions

Following the ORT experiments rats were given access to 24-hour chronic intermittent ethanol sessions 3 days/week for 4 weeks using a three-bottle choice paradigm. The sessions began at the beginning of the dark cycle. The rats were weighed at the beginning of each session and received 3 bottles containing either water, 15% EtOH, or 30% EtOH. The bottles were removed 24 hours later and weighed to measure ethanol or water consumption. Repeated-measure ANOVA was used to assess the effect of sex on ethanol consumption and total fluid intake. Linear regression models were used to assess potential correlations between novelty-seeking behaviors and alcohol consumption levels.

RESULTS

First, we assessed the exploratory behaviors (i.e. time spent in each quadrant, time spent investigating the objects and the total number of rears) of male and female HAD-1 rats during the training phase when they are first introduced to the test chamber. Next, we assessed the same exploratory behaviors during the subsequent testing phase, in addition to the time spent investigating the novel vs. the familiar object. Lastly, we assessed the

relationship between average daily alcohol consumption during the 5-week chronic ethanol exposure sessions and novelty-seeking behaviors.

Training Phase

During the initial training phase, female HAD-1 rats spent significantly more time in the object-containing quadrant (Figure 1A; $t_{14} = 4.514$, $p < 0.001$) and also spent more time exploring the objects (Figure 1C; $t_{14} = 3.914$, $p < 0.01$) as compared to male HAD-1 rats. No significant sex differences were observed in the total time spent in the other quadrants (Figure 1B). Moreover, there was a significant Sex*Quadrant interaction (Figure 1D; $F_{3,56} = 4.926$, $p < 0.01$) and a main effect of sex ($F_{1,56} = 8.923$, $p < 0.01$) on the total number of rears. Post-hoc analyses revealed that female HAD-1 rats made more rears than male HAD-1 rats in the first (object-containing; $t_{56} = 3.414$, $p < 0.001$) and the fourth quadrants ($t_{56} = 3.181$, $p < 0.001$).

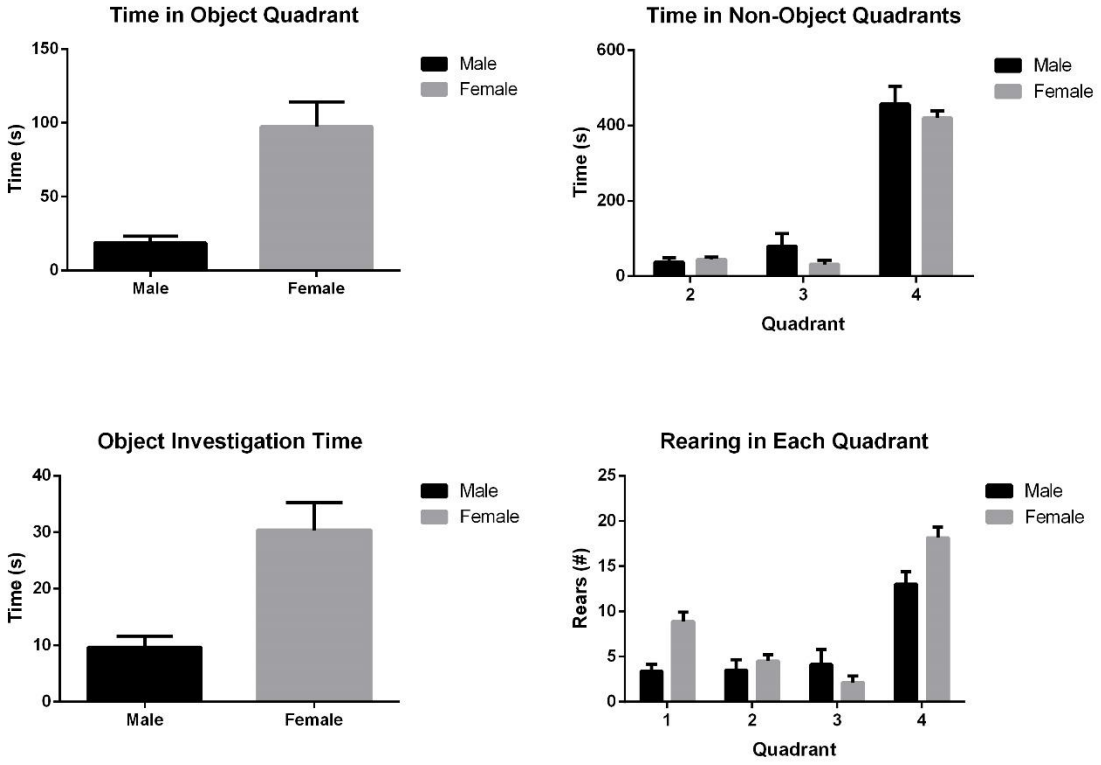
Testing Phase

During the subsequent testing phase, female HAD-1 rats only showed a marginal increase in the amount of time spent in the object-containing quadrant (Figure 2A; $t_{14} = 2.063$, $p = 0.0582$) as compared to male HAD-1 rats. No further sex differences were observed in the time spent in other quadrants (Figure 2B), time spent investigating the objects (Figure 2C) or total number of rears made during the test session (Figure 2D). While both male and female HAD-1 rats spent more time investigating the novel object (male: 10.88 ± 3.32; female: 17.75 ± 3.05) than the familiar object (male: 7.00 ± 1.89; female: 10.38 ± 2.86), there were no sex differences in object recognition between these rats.

Ethanol Consumption

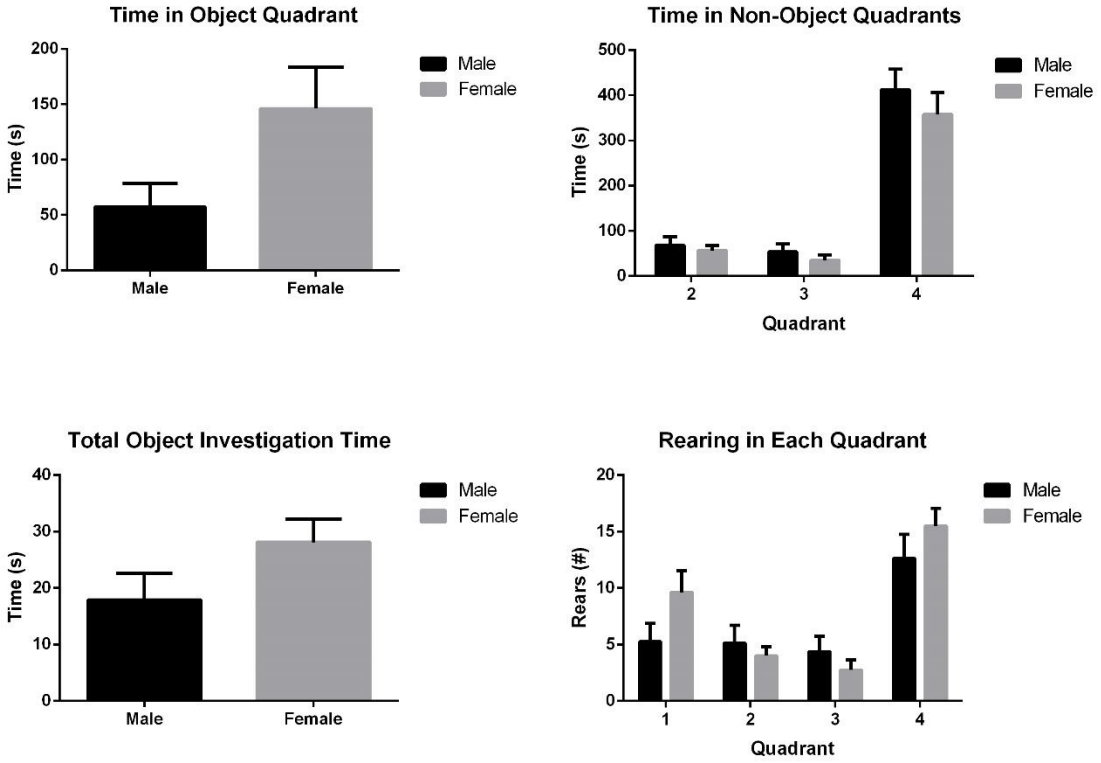
Female HAD-1 rats consumed significantly higher amounts of alcohol than the male rats throughout the 5-week ethanol availability experiment (Figure 3A; $F_{1,195}$ = 70.08, $p < 0.0001$). There were no sex differences in total fluid consumption during the ethanol availability experiments (Figure 3B). Linear regression modeling showed a significant correlation between alcohol consumption and the object investigation time (Figure 3C; $F_{1,13}$ = 13.08, $p < 0.01$), as well as, rearing behavior (Figure 3D; $F_{1,13}$ = 6.74, $p < 0.05$) during the training phase.

Figure 6: Sex differences in exploratory activity during the sample trial of ORT.



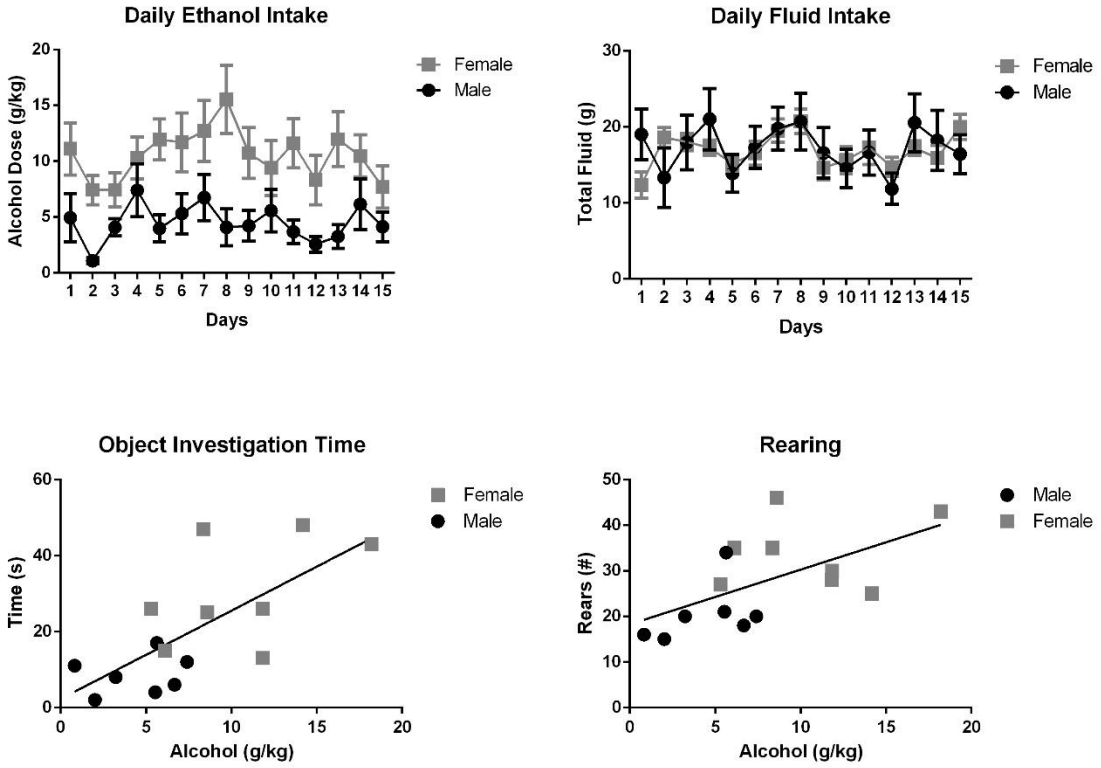
A) Female rats spent more time exploring the object quadrant than male rats ($p < 0.001$). B) There were no sex differences in the time spent in the other three quadrants. C) Female rats spent more time exploring the objects than male rats ($p < 0.01$). D) There was a significant Sex*Quadrant interaction ($p < 0.01$) and a main effect of sex ($p < 0.01$) on rearing behaviors during the sample trial. Post-hoc analysis revealed that female rats made more rears in than male rats in first ($p < 0.001$) and the fourth ($p < 0.001$) quadrants.

Figure 7: Sex differences in exploratory activity during the test trial of ORT.



A) Female rats spent more time exploring the object quadrant than male rats ($p = 0.058$). There were no further sex differences in B) the time spent in the other three quadrants, C) time exploring the objects, or D) rearing behaviors during the test trial.

Figure 8: ORT exploratory activity predicts future alcohol consumption.



A) Female rats drank significantly more alcohol than male rats ($p < 0.0001$). B) There were no sex differences in total fluid intake. C) There was a significant correlation between object investigation time during the sample trial and total alcohol consumption ($p < 0.01$). D) There was a significant correlation between total rearing during the sample trial and total alcohol consumption ($p < 0.05$).

DISCUSSION

In this study we examined potential sex differences in novelty seeking, object recognition and ethanol consumption between male and female HAD-1 rats. Here we report that female rats show increased exploratory activity, as measured by rearing behavior and object investigation time, during the initial training phase of ORT. Female HAD-1 rats also consume substantially more alcohol than their male counterparts. Moreover, there was a significant of correlation between object investigation time, total rearing behavior and alcohol consumption in these rats. Together these results provide evidence that novelty-seeking behavior within the ORT can serve as a predictor for the propensity to consume excess alcohol in male and female HAD-1 rats.

Novelty-seeking behaviors seems to be a good marker for the initiation of drug-taking behavior (Piazza et al., 1989), as well as the subsequent tendency for compulsive use and relapse (Flagel et al., 2014). Rats with high levels of novelty-induced activity express higher levels of galanin and enkephalin in the paraventricular nucleus and orexin in the peri-fornical lateral hypothalamus (Barson et al., 2013). Moreover, high levels of rearing in a novel chamber are associated with lower levels of neurotensin in the paraventricular nucleus of the thalamus (Pandey et al., 2017). While ORT was initially developed primarily as a memory task (Ennaceur and Delacour, 1988), exploratory behaviors during the training phase can reveal novelty-seeking phenotypes (Dere et al., 2007). Hippocampal lesions have been shown to reduce object exploration during the sample phase of ORT (Ainge et al., 2006), and decreased cholinergic activity is associated with a decrease in exploratory activity as the environment becomes familiar (Inglis et al., 1994). Although we did not directly explore these neural substrates in the present study, the differences in novelty seeking and rearing behaviors observed here might be due to potential differences in neural pathways regulating attention and arousal

in male and female HAD-1 rats. Our findings provide the rationale for future studies to directly explore these pathways in HAD-1 rats.

Converging lines of evidence show that female rats typically consume higher levels of alcohol than their male counterparts (Li and Lumeng, 1984; Maldonado-Devincci et al., 2010). However, the finding in the present work showing higher levels of ethanol consumption in female HAD-1 rats contradicts with previous work showing no sex-differences in alcohol consumption in adult HAD-1 rats (Dhaher et al., 2012). It is possible that procedural differences between the previous study and the present study might explain the contradictory findings. For instance, the previous study utilized a two-bottle choice paradigm with continuous 22-hr per day access to 15% ethanol for 30 days, whereas our study used a three-bottle choice design (water, 15% and 30% ethanol) and only provided access to ethanol 3 days per week for 5 weeks. The female HAD-1 rats exhibited greater levels of attention as indicated by the increased time spent investigating novel objects. Therefore it is possible that the presence of an extra-bottle and the disruption associated with the removal and reintroduction of the bottles may differentially favor increased consumption in female rats because they may be more sensitive to environmental disruptions and be more likely to investigate the source. This hypothesis will need to be directly tested in future studies, to address the sex-dependent discrepancies in alcohol consumption observed here.

In summary, the present work provides novel evidence of increased exploratory and novelty-seeking behaviors in female HAD-1 rats as compared to their male counterparts. Moreover, we show that these differences in novel object investigation and rearing behaviors are in line with and predictive of increased alcohol consumption behaviors in rodents. These behavioral outcomes may represent functional differences in neurochemical substrates underlying alcohol and drug-seeking behaviors. These results

highlight the need for direct examination of these systems in male and female HAD-1 rats.

Chapter 4: Alcohol-Naïve USVs Distinguish Male HAD-1 from LAD-1 Rat Strains [3]

ABSTRACT

Ultrasonic vocalizations (USVs) are mediated through specific dopaminergic and cholinergic neural pathways and serve as real-time measures of positive and negative emotional status in rodents. Although most USV studies focus primarily on USV counts, each USV possesses a number of characteristics shown to reflect activity in the associated neurotransmitter system. In the present study, we recorded spontaneously emitted USVs from alcohol-naïve high alcohol drinking (HAD-1) and low alcohol drinking (LAD-1) rats. Using our recently developed WAAVES algorithm we quantified four acoustic characteristics (mean frequency, duration, power and bandwidth) from each 22 – 28 kHz and 50 – 55 kHz frequency modulated (FM) USV. This rich USV representation allowed us to apply advanced statistical techniques to identify the USV acoustic characteristics that distinguished HAD-1 from LAD-1 rats. Linear mixed models (LMM) examined the predictability of each USV characteristic in isolation and linear discriminant analysis (LDA) and binomial logistic regression examined the predictability of linear combinations of the USV characteristics as a group. Results revealed significant differences in acoustic characteristics between HAD-1 and LAD-1 rats in both 22 – 28 kHz and 50 – 55 kHz FM USVs. In other words, these rats selectively bred for high- and low-alcohol consumption can be identified as HAD-1 or LAD-1 rats with high classification accuracy (approx. 92-100%) exclusively on the basis of their emitted 22-28 kHz and 50-55 kHz FM USV acoustic characteristics. In addition, acoustic

[3] Reprinted from Mittal, N., Thakore, N., Reno, J. M., Bell, R. L., Maddox, W. T., Schallert, T., & Duvauchelle, C. L. (Copyright 2017). Alcohol-Naïve USVs Distinguish Male HAD-1 from LAD-1 Rat Strains. Alcohol. https://doi.org/10.1016/j.alcohol.2017.09.003, with permission from Elsevier. NM was responsible for experiment design, data collection, analysis, interpretation, and writing of this manuscript.

characteristics of 22 – 28 kHz and 50 – 55 kHz FM USVs emitted by alcohol-naïve HAD-1 and LAD-1 rats significantly correlate with their future alcohol consumption. Our current findings provide novel evidence that USV acoustic characteristics can be used to discriminate between alcohol-naïve HAD-1 and LAD-1 rats, and may serve as biomarkers in rodents with a predisposition for, or against, excessive alcohol intake.

INTRODUCTION

Drug addiction is a chronic relapsing disorder with a strong emotional component. During initial use, drugs of abuse hijack the midbrain reward system to produce euphoria and heightened positive emotional states (Wise and Koob, 2014). Persistent or chronic use of these drugs results in a shift in the baseline homeostatic activity of these systems and results in the emergence of a negative affective or withdrawal state when the drug is no longer present (Koob and Volkow, 2016). The onset of this negative state is an important aspect of the transition from recreational drug use to drug dependence. Moreover, individuals with pre-existing negative affective states either due to depression (Schuckit et al., 2006; Conner et al., 2009), posttraumatic stress (Gilpin and Weiner, 2016), or early life adversity (Cornelius et al., 2016) are likely to engage in relapse like behaviors (Watkins et al., 2015) which can further increase their risk of developing a substance use disorder (SUD). Furthermore, strategies aimed at improving emotional regulation have shown promise in reducing drug abuse behaviors (Tang et al., 2016). Together these studies highlight an important need for understanding the role of emotion in promoting hazardous drug use.

Emotion has been described as a complex psychological state with three components: i) a subjective experience, ii) an underlying neural substrate, and iii) an expressive/behavioral and/or autonomic response (Chiurchiù and Maccarrone, 2016). The

clinical studies described above show a clear relationship between the subjective experience (internal and/or external) produced by alcohol and other drugs of abuse and the resulting emotional response. However, since there are few reliable pre-clinical models of emotion, the neural substrates that underlie these phenomena are not well understood.

Ultrasonic vocalizations (USVs) have been identified as real-time functional measure of emotional status in rodents (Brudzynski, 2009, 2013). Converging evidence from ethological, pharmacological, and neuroanatomical studies has shown that 22 – 28 kHz USVs occur in response to alarm, punishment, or avoidance behaviors and typically represent negative affective status; while 50 – 55 kHz frequency modulated (FM) USVs, directly evoked by dopamine release (Scardochio et al., 2015) and produced in response to rewarding stimuli including food, drugs, or sex are thought to represent positive affective states (Knutson et al., 2002). Moreover, each USV is multidimensional and is characterized by a rich set of acoustical properties, including frequency (kHz), duration, bandwidth and power. 22 – 28 kHz USV counts and acoustic characteristics can be directly regulated by cholinergic agonists and antagonists (Brudzynski and Bihari, 1990; Brudzynski, 2001) and 50 – 55 kHz FM USVs can be directly regulated by activating (Maier et al., 2012; Ahrens et al., 2013) or inhibiting (Wintink and Brudzynski, 2001; Williams and Undieh, 2010) the dopaminergic system. Other neurotransmitter systems also shown to modulate USV activity include the neurotensin (Prus et al., 2014; Steele et al., 2017), 5-HT (Beis et al., 2015; Wöhr et al., 2015) and adenosine (Simola et al., 2016) systems. Therefore, spontaneous baseline USV activity may relay important information about underlying neurotransmission.

Animal models of high alcohol consumption reveal an intimate relationship between USVs and propensity for excessive drinking. For example, the selectively bred

alcohol preferring (P) and alcohol non-preferring (NP) rats are established rodent models of high alcohol drinking and alcohol avoidance, respectively. The high-alcohol-drinking (HAD-1) and low-alcohol-drinking (LAD-1) rats are another such model that satisfy many of the criteria for an animal model of alcoholism, such as high levels of alcohol consumption during adolescence and adulthood, pronounced alcohol seeking behaviors, and an alcohol deprivation effect under relapse conditions (Bell et al., 2014; McBride et al., 2014). In two studies from our laboratory we found that P and HAD-1 rats spontaneously emit significant numbers of negative affect USVs even in the alcohol-naïve state (Reno et al., 2015; Thakore et al., 2016).

Our recent development of a MATLAB-based algorithm (WAAVES) (Reno et al., 2013; Reno and Duvauchelle, 2014) automates the tabulation of USV counts and acoustic characteristics, thereby allowing us to conduct long term studies exploring counts and acoustic characteristics of spontaneously emitted USVs over multiple recording sessions. Using this tool, we conducted a study focused just on P and NP rats (Reno et al., 2017) and found that alcohol-naïve P and NP rats can be distinguished based solely on the acoustic properties associated with 22 – 28 kHz USVs. The ability to distinguish between high- and low-drinking lines according to USV profiles suggests that drinking propensity and USV emissions may be regulated by common neural substrates. The present study aims to extend our previous findings and examine whether either positive or negative affect-associated USV acoustic properties can similarly be used to distinguish between alcohol-naïve HAD-1 and LAD-1 rats.

This work embraces the multidimensional nature of each USV and subjects these USVs to multivariate statistical procedures including linear mixed modeling, linear discriminant analysis and binomial logistic regression. Linear mixed modeling has a number of advantages over more traditional ANOVA based approaches. For example,

rather than using individual or group means, each and every USV acoustic characteristic is input into the analyses, resulting in a full representation of all data. The goal of linear discriminant analysis is to estimate the linear "discriminant" that best separates the multidimensional USVs associated with two groups of rats. In essence, multivariate data is linearly combined to produce a univariate variable aimed at separating groups. We use a 10,000-iteration training-test bootstrapping procedure to fit the model and to determine whether the percent of animals correctly classified by linear discriminant analysis is significantly above chance. Binomial logistic regression is similar in spirit to linear discriminant analysis in that the goal is to discriminate two groups of rats from a linear combination of the USV acoustic characteristics. The difference is that binomial logistic regression makes fewer assumptions regarding the nature of the underlying distributions. By including both linear discriminant analysis and binomial logistic regression we can look for convergence in the conclusions drawn.

Using these powerful analytic tools, the goal of this study was to determine whether HAD-1 and LAD-1 rats could be distinguished solely from the acoustic characteristics associated with spontaneous USVs emitted in the alcohol-naïve state. In this study, linear mixed modeling was used to assess whether the mean frequency, duration, bandwidth, or power of 22 – 28 kHz and 50 – 55 kHz FM calls differed significantly between the HAD-1 and LAD-1 rat lines. Next, we used linear discriminant analysis to determine whether a linear combination of these four acoustic characteristics could be used to distinguish HAD-1 from LAD-1 rats. Lastly, we cross-validated the LDA results using binomial logistic regression.

MATERIALS AND METHODS

Subjects

Animals

Male high alcohol drinking rats (n = 6; HAD-1 rats: generation 65) and low alcohol drinking rats (n = 6; LAD-1 generation 64) were obtained from Indiana University School of Medicine, Indianapolis, IN, at approximately 32 days old. Animals were handled 5 days per week for 4 weeks to habituate them to experimenters. The University of Texas Institutional Animal Care and Use Committee (IACUC) approved all housing and experimental procedures.

Ultrasonic Vocalization Recordings

HAD-1 and LAD-1 rats were recorded under alcohol-naïve conditions. Following the habituation period, USVs were recorded in 4 hour sessions for 3 days/week for 4 weeks. CM16 microphones were used with an UltraSound Gate interface (Avisoft Bioacoustics) to record USVs at a 250-kHz sampling rate and a 16-bit resolution. On recording days, animals were weighed at the beginning of the dark cycle, transported to a test room, and placed into recording cages (which were identical to their home cage but only used for USV recordings) for 4h test sessions. Each animal was assigned its own recording cage in order to prevent any non-specific behaviors related to novelty or conspecific scents (Wöhr et al., 2008). Based on rat and chamber size, we approximate the distance between the animal's head and the centered microphone to range from 5 cm to 28.4 cm. After the recording session, the animals were transported back to the vivarium and returned to their home cage.

Analysis of USVs

Ultrasonic vocalization recordings were analyzed using the WAAVES program (Reno et al., 2013; Reno and Duvauchelle, 2014). This program reads audio files and produces a frequency spectrogram. The spectrogram is then scanned for sound objects using MATLAB's *Image Processing Toolbox* (MathWorks, Inc. Natick, MA). For 50 – 55 kHz FM USVs, WAAVES identifies sound objects with a minimum duration of 5 ms occurring in a range of 30 – 120 kHz. An inter-call interval of 10 ms was used to discriminate between individual calls and avoid counting call fragments as separate calls. FM USVs were defined as calls that varied more than 5 kHz over the entire duration of the call. 22 – 28 kHz calls were identified as sound objects occurring in a frequency range of 20 to 30 kHz with a minimum duration of 200 ms. An inter-call interval of 100 ms was used to separate individual calls. These call parameters were derived from the existing literature as well as extensive trial-and-error testing in the laboratory. Some preliminary tests of the robustness of these parameters were undertaken during development of the WAAVES algorithm. Generally speaking, the results were robust to small changes in the WAAVES parameters. Once the calls are identified, several measurements of interest are extracted from each USV call and stored for subsequent analysis. The mean frequency, duration, bandwidth, and power for both 50-55 kHz FM and 22-28 kHz calls were used for statistical analysis.

Validation Process for WAAVES Automation.

Validation of WAAVES-generated USV data requires correspondence with human-derived analyses. Experimenters manually analyzed subsets of USV data recorded during the experiment to compare human assessment with WAAVES output. USV data subsets used for manual validation consisted of 80 (out of 3456) 10-min USV files recorded from HAD and LAD rats. The total number of calls identified via manual

analysis was correlated with the total number of calls identified by the automated WAAVES program. Separate correlations were also conducted for each group (i.e. HAD and LAD) in order to confirm comparable findings across rat lines. The correlation coefficients are reported in the results.

EtOH Drinking Sessions

Following the USV recording experiments the rats received chronic intermittent access to three bottle choice alcohol (water, 15% EtOH, 30% EtOH) in the home cage, 24 hrs/day, 3 days/week (e.g. Monday, Wednesday, Friday) for 4 weeks in order to validate high vs low alcohol consumption between the selectively bred HAD-1 and LAD-1 rats.

Statistical Approach

A standard statistical approach would utilize repeated measures ANOVA to analyze the USV data. In this approach, all calls emitted by a rat are used to calculate an average, and then any potential group differences in these averages are assessed. Thus, this method results in loss of important information pertaining to the inter-individual variability in USV calls for each rat, which, in turn, reduces power. To overcome these problems, we used linear mixed models to examine the effect of selective breeding (e.g. HAD-1 vs LAD-1) on total USV counts and the pattern of USV acoustic characteristics (e.g. mean frequency, duration, bandwidth, or power). Linear mixed models allow us to use the data from all the calls emitted by each rat, and can also assess for random day-to-day variation due to repeated measurements even in the event of missing data at any of the time points measured. If a significant group effect was observed, its impact on the model's goodness of fit was tested by creating a reduced null model without the group, and then by comparing the reduced model with the full model using an ANOVA. The p-values resulting from the ANOVA are also reported.

Linear Mixed Models: We assessed differences in total USV counts and each of the four USV characteristics as a function of rat line using a linear mixed model in R (R Core Team, 2015) using the package "lmerTest" (Kuznetsova et al., 2016). The linear models were generated for each experimental group for each of the 4 acoustic characteristics of interest. The models were used to assess the effect of time, rat line, or an interaction of these factors on each of these characteristics. Whenever a significant effect was observed a new reduced model was generated by removing the significant factor and compared with the original model using an ANOVA in order to assess the impact of the respective factor on the goodness-of-fit for the model. The resulting model is a regression equation where the intercept and slope is allowed to vary for each rat:

$$Y_{Acoustic\ Characteristic} = \beta_0 + \beta_{Rat\ Line} X_{Rat\ Line} * \beta_{Set\ Day} X_{Set\ Day} + W_{Rat} + U_{Rat*Set\ Day}$$

where $Y_{Acoustic\ \ Characteristic}$ is the acoustic characteristic being modeled (e.g. mean frequency, duration, bandwidth, or power), each predictor variable is represented by its subscripted X, W_{Rat} represents the random effect of each individual rat, and $U_{Rat*Set\ Day}$ represents the random effect of day to day variation for each rat. A random slope coefficient was included to protect against potential noise introduced by random day-to-day variation in call parameters for each rat. The coefficients (β) are estimated and assessed for significance, as if so, the contribution to the goodness of fit of the model was assessed.

Linear Discriminant Analysis: LMM focuses on each acoustic property in isolation. To assess the combined interactive effect of all four USV characteristics we applied linear discriminant analysis (LDA) using the R package "MASS" (Venables and Ripley, 2002) to determine if a linear combination of these data was capable of distinguishing the rat lines (e.g., HAD-1 vs LAD-1). A linear combination of the multivariate data is used to calculate a univariate (discriminant) value that represents the

maximum separation between the groups. Thus, the LDA can be used to determine whether USVs, across acoustic characteristics, emitted by alcohol-naïve HAD-1 rats differ from those emitted by alcohol-naïve LAD-1 rats. Because we were interested in examining the ability of these acoustic characteristics to distinguish rat lines, we assessed all USVs emitted by each group (e.g. HAD-1 rats and LAD-1 rats) without reference to time.

Since the data are used in building the model, it is possible that the best fitting model would be specific to the data used and may not necessarily generalize to the population as a whole. To address this issue and ensure the generalizability of the model, we split the data into a training and testing subset; where one half of the animals are used to train the model and the remaining half are used to test it. When dividing the groups into training and testing subsets, it is possible that certain combinations of animals within each subset may be more (or less) representative of the entire dataset and, in turn, bias the ability of the model to accurately separate the groups. Thus, in order to produce an accurate assessment, we repeated the LDA 10,000 times, each time randomly selecting half of the data as our training set and using the remaining half to test the model. We then computed the percent of animals correctly assigned to their group[4] for each of the 10,000 iterations. The resulting distribution allows us to estimate the average percent correct and

[4] To compute the percentage of animals correctly assigned to their groups by the LDA, we first computed the average LDA value across all USVs emitted by each animal. Next, we combined the average USV LDA values for each animal to compute the group averages for HAD-1 and LAD-1 rats. We then calculated the midpoint between these two means and used this midpoint as the decision boundary for separation. The animals were then classified as HAD-1 or LAD-1 based on the side of the decision boundary on which their LDA values clustered.

standard error for each iteration, thereby allowing us to compute 95% confidence intervals around the mean percent correct for the 10,000 trials. If the model performs no better than chance alone, we would expect 50% of the animals to be correctly categorized. Therefore, if the 95% confidence interval around the average percent correct includes 50% we cannot conclude that the model is performing better than chance at an alpha level of 0.05.

Binomial Logistic Regression: Binomial logistic regression was performed similar to the LDA. The data were randomly split into two groups; one group was used to train the model and the other group was used to test the classification accuracy of the model. Unlike the LDA that produces linear discriminant coefficients, the logistic regression provides probability values for whether a call belongs to a HAD-1 or LAD-1 rat. Similar to the LDA, we then averaged the call probabilities to obtain the average probability for each rat. These rat probabilities were then grouped by HAD-1 or LAD-1 and the midpoint between the probabilities was used as the decision boundary for separation. This process was also repeated 10,000 times and the mean classification accuracy and 95% confidence interval for these iterations are reported.

RESULTS

Linear Mixed Models

22 – 28 kHz USVs

We began by examining differences in USV counts. Alcohol-naïve HAD-1 (total call counts = 854.83 ± 259.71) and LAD-1 (total call counts = 613.33 ± 289.52) rats spontaneously emitted 22 – 28 kHz USVs during the 4-hour recording sessions. However, no significant effect of rat line was observed on the total number of calls emitted. Next, we examined the USV acoustic properties. We observed significant

group*day interactions on the mean frequency ($p < 0.0001$, $t_{4576} = 3.974$; Figure 1a), duration ($p < 0.0001$, $t_{165.4} = 4.591$; Figure 1b) and power ($p < 0.001$, $t_{6656} = -3.319$; Figure 1d), but not the bandwidth ($p = 0.146$, $t_{133.6} = -1.461$; Figure 1c) of 22 – 228 kHz USVs. Removal of the interaction significantly reduced the goodness-of-fit for the model for each of the three parameters: mean frequency ($p < 0.0001$, $\chi^2 = 15.397$), duration ($p < 0.0001$, $\chi^2 = 16.333$), and power ($p < 0.01$, $\chi^2 = 10.751$). However, visual analysis did not reveal any clear group*day trends. It is possible that theses test may be too sensitive to the within-subject day-to-day variability observed in USV calls. Therefore, the main effect of group was also analyzed for each acoustic characteristic whether or not a group*day interaction was observed. We found a significant effect of rat line on the duration ($p < 0.0001$, $t_{2222} = -25.02$; Figure 1b), and power ($p < 0.0001$, $t_{7888} = 4.946$; Figure 1d) of 22-28 kHz USVs of alcohol-naïve HAD-1 and LAD-1 rats. No such effect of rat line was observed on the mean frequency ($p = 0.303$, $t_{6894} = 1.03$; Figure 1a) or bandwidth ($p = 0.151$, $t_{4142} = 1.438$; Figure 1c). Removal of the group effect resulted in a significant reduction in the goodness-of-fit of the linear mixed model for both the duration ($p < 0.0001$, $\chi^2 = 592.38$) and power ($p < 0.0001$, $\chi^2 = 24.06$).

In summary, there were statistically significant group*day interactions in the mean frequency, duration and power, but not the bandwidth of 22 – 28 kHz USVs, although the directionality of these interactions was not apparent through visual analysis. We also observed a significant effect of rat line between alcohol-naïve HAD-1 and LAD-1 rats on the duration and power of 22 – 28 kHz calls. Post-hoc analyses did not reveal any clear differences in the call power of 22-28 kHz USVs between HAD-1 and LAD-1 rats, the LAD-1 rats made longer calls than HAD-1 rats on all recording days over the 4-week period. No further effects were observed on the mean frequency or bandwidth of 22 – 28 kHz USVs.

Next, we used regression analyses to determine whether the USV acoustic properties of 22 – 28 kHz calls corresponded with future alcohol consumption in these rats. We found a significant negative correlation between the duration of 22 – 28 kHz calls and future EtOH consumption ($R = -0.866$, $p < 0.01$) in the combined HAD-1 and LAD-1 sample. No further correlations were observed between EtOH consumption and the mean frequency, bandwidth or power of 22 – 28 kHz calls.

50 – 55 kHz USVs

We again began with an examination of USV counts and followed with analyses of the USV acoustic characteristics. Both HAD-1 (total call counts = 272 ± 44.84) and LAD-1 (total call counts = 168.17 ± 34.38) rats emitted spontaneous 50 – 55 kHz FM USVs. There was a significant effect of rat line on the total number of USVs emitted ($p < 0.05$, $t_{102.05} = 2.396$). Removing the effect of rat line resulted in a significant reduction in the goodness-of-fit of the model ($p < 0.05$, $\chi^2 = 5.3354$). There were no significant group*day interactions for the mean frequency ($p = 0.212$, $t_{68.270} = -1.259$), duration ($p = 0.872$, $t_{14,722} = -0.164$), bandwidth ($p = 0.602$, $t_{19.906} = -0.529$) or power ($p = 0.108$, $t_{27.270} = -1.663$) of 50 – 55 kHz FM calls. However, significant effects of rat line were observed in the mean frequency ($p < 0.0001$, $t_{289.31} = 9.896$; Figure 2a), duration ($p < 0.0001$, $t_{18.779} = 14.72$; Figure 2b), bandwidth ($p < 0.0001$, $t_{79.790} = -5.248$; Figure 2c), and power ($p < 0.01$, $t_{204.19} = -3.18$; Figure 2d) of 50 – 55 kHz FM USVs of alcohol-naïve HAD-1 and LAD-1 rats. Removing this effect significantly reduced the goodness-of-fit for the model regarding each of the four parameters: mean frequency ($p < 0.0001$, $\chi^2 = 89.31$), duration ($p < 0.0001$, $\chi^2 = 117.48$), bandwidth ($p < 0.0001$, $\chi^2 = 25.963$), and power ($p < 0.01$, $\chi^2 = 8.3959$).

In summary, although we did not see any group*day interaction in any of the characteristics measured, there were statistically significant group differences between alcohol-naïve HAD-1 and LAD-1 rats in USV counts, mean frequency, duration, bandwidth and power of 50 – 55 kHz FM calls. The HAD-1 rats made calls with a higher mean frequency and longer duration than the LAD-1 rats, while the LAD-1 rats made calls with a wider bandwidth. The effect of rat line on USV dB levels (e.g., power) of these calls was not clear.

Regression analysis was used to determine whether the USV acoustic properties of 50 – 55 kHz FM calls corresponded with future alcohol consumption in these rats. We found a significant positive correlation between future EtOH consumption and the mean frequency ($R = 0.690$, $p < 0.05$) and duration ($R = 0.899$, $p < 0.001$) of 50 – 55 kHz FM USVs in the combined sample. In addition, a significant negative correlation was observed between EtOH consumption and the bandwidth ($R = -0.815$, $p < 0.01$) of 50 – 55 kHz FM calls in the combined sample. There was no significant correlation between EtOH consumption and the power of 50 – 55 kHz FM calls emitted by these rats.

Linear Discriminant Analysis

After assessing the differences between HAD-1 and LAD-1 rats on total emitted calls and each individual acoustic characteristic using linear mixed models, we sought to examine whether it was possible to discriminate these groups by using a combination of the mean frequency, duration, bandwidth, and power of USV calls. One way to achieve this is to use a linear discriminant analysis, a statistical and machine-learning method used to separate two or more classes of objects (e.g. HAD-1 vs. LAD-1) based on a linear combination of explanatory variables. To achieve this aim, we split our data into "testing and "training" subsets and used the bootstrapping approach described in the statistical

methods above. Once we were confident that the LDA model could accurately classify the two strains we generated a new equation using the entire data set in order to calculate the coefficients associated with each acoustic characteristic.

22 – 28 kHz USVs

The LDA equation calculated using the mean frequency, duration, bandwidth, and power of 22 – 28 kHz USVs from alcohol-naïve rats resulted in perfect characterization of HAD-1 and LAD-1 rats in 3,674 of the 10,000 iterations. The mean classification accuracy was 81.96%, and the 95% confidence interval was 50% - 100%. Though it should be noted that 9,283 out 10,000 iterations produced classification accuracy greater than 66.66%. For the LDA equation the order of the degree of separation contributed by each of the acoustic characteristics was as follows: call duration, power, bandwidth, and mean frequency. With call duration contributing the most to the separation and mean frequency contributing the least. Applying LDA to the full complement of the data resulted in a maximum separation accuracy of 91.66% (Figure 3a). The corresponding equation coefficients are listed in the table below (Table 1).

50 – 55 kHz USVs

The LDA equation calculated using the mean frequency, duration, bandwidth, and power of 50 – 55 kHz USVs from alcohol-naïve rats resulted in perfect characterization of HAD-1 and LAD-1 rats for all 10,000 iterations. Therefore, the mean classification accuracy was 100% and the 95% confidence interval was the same (Figure 3a). The order of contribution to the separation capacity of the model was: call duration, mean frequency, power, and bandwidth. Applying the LDA to the full complement of data also resulted in complete separation of the two rat lines. The corresponding equation coefficients are listed in the table below (see Table 1).

Binomial Logistic Regression

While linear discriminant analysis is a well-established method of classifying a binary data set (such as the HAD-1 vs. LAD-1 data) using independent predictor variables (such as USV acoustic characteristics), it relies on the assumption that these predictor variables are normally distributed. In order to test the distribution of our data we performed a Shapiro-Wilk normality test and found that none of the four variables of interest had a normal distribution. Although small deviations in normality are not thought to significantly impact the outcome of LDA, the lack of normality in our data highlighted the need to further validate the results achieved with the LDA approach using a second method. Binomial Logistic Regression is another such technique that can be used to develop linear classification models, which relies on fewer assumptions than the LDA method (Pohar et al., 2004). Thus, the logistic regression approach might be better suited in instances where the assumptions of the LDA are violated. We performed logistic regression in a manner similar to the LDA method described above. The data were split into testing and training subsets and the classification accuracy was measured. The process was repeated 10,000 times, and the mean and its 95% confidence interval for classification accuracy are reported.

22 – 28 kHz USVs

The binomial logistic regression equation calculated using the mean frequency, duration, bandwidth, and power of 22 – 28 kHz USVs from alcohol-naïve rats resulted in perfect characterization of HAD-1 and LAD-1 rats in 5,044 of the 10,000 iterations. The mean classification accuracy was 91.74%, and the 95% confidence interval was 83.33% - 100%. Similar to the LDA results when logistic regression was applied to the full complement of data, a separation accuracy of 91.66% was achieved (Figure 3b). The corresponding logistic equation coefficients are reported in the table below (see Table 2).

50 – 55 kHz USVs

The binomial logistic regression equation calculated using the mean frequency, duration, bandwidth, and power of 50 – 55 kHz USVs from alcohol-naïve rats also resulted in perfect characterization of HAD-1 and LAD-1 rats for all 10,000 iterations. As such, the mean classification accuracy was also 100% and the 95% confidence interval was the same (Figure 3b). The corresponding logistic equation coefficients are reported in the table below (Table 2).

Together, these results show that the logistic regression approach is indeed more robust than the LDA approach in classifying "unseen" data. However, when applied to the complete dataset, the LDA provides similar accuracy. Thus, the BLR provides strong confirmatory support for the present LDA results.

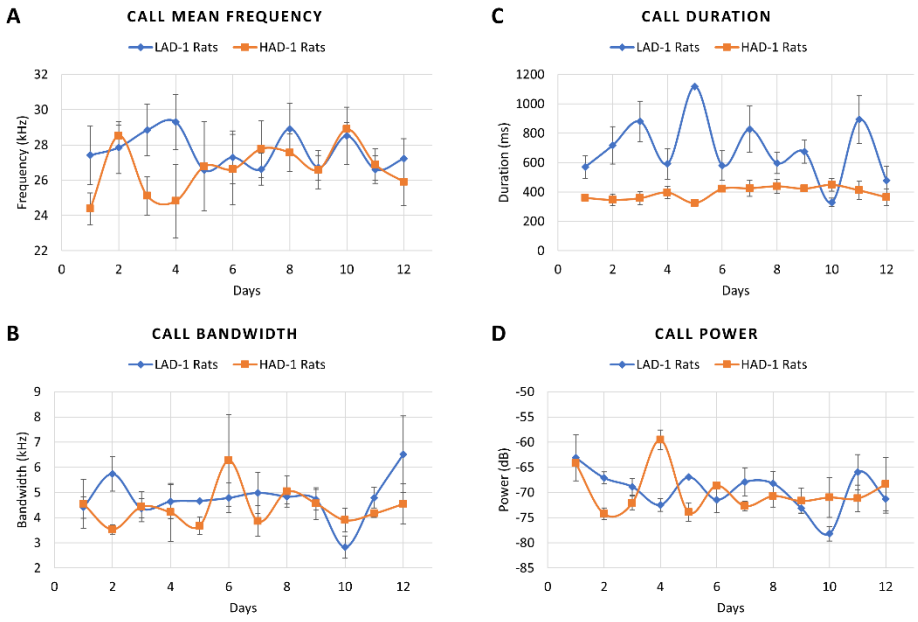
Alcohol Consumption

Repeated-measures ANOVA revealed a significant group by time interaction for EtOH consumption levels ($p < 0.001$, $F_{11,110} = 3.72$; Figure 4a). Pearson's correlation analysis was used as a post-hoc measure to further explore the group by time interaction. As expected, escalation in alcohol intake was observed over time in HAD-1 rats ($r = 0.387$, $p < 0.001$; Figure 4b), but not in LAD-1 rats ($r = -0.153$, $p = 0.20$).

Validation of USV Analysis

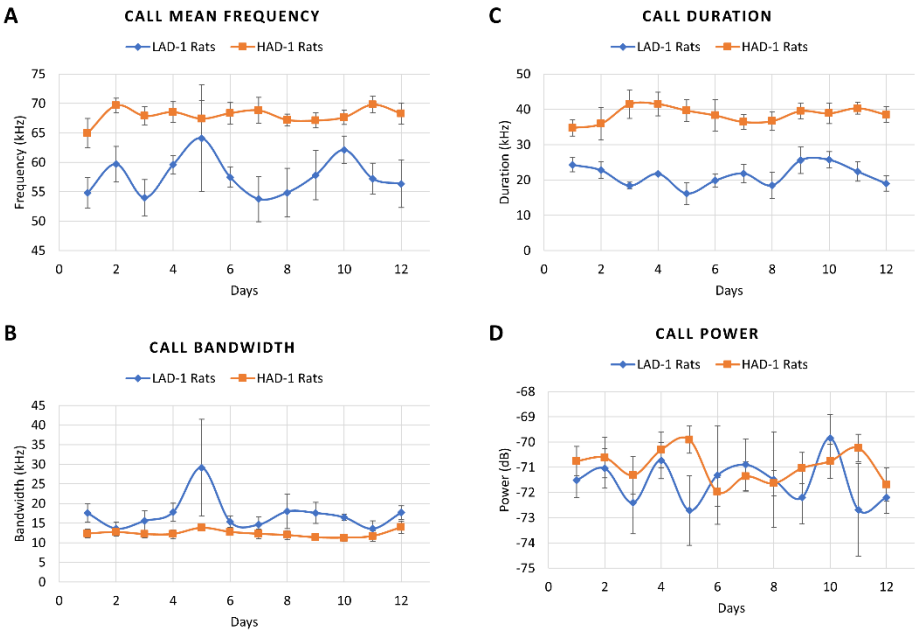
WAAVES-automated analysis and manual analysis results were highly correlated for both the 22 – 28 kHz calls ($r = 0.996$) and 50 – 55 kHz ($r = 0.936$). Correlation analyses run separately for HAD and LAD 22-28 and 50-55 kHz USVs showed comparable high correspondence across lines between WAAVES and human-derived counts (HAD: 22 – 28 kHz: $r = 0.997$; 50 – 55 kHz: $r = 0.940$; LAD: 22 – 28 kHz: $r = 0.999$; 50 – 55 kHz: $r = 0.946$).

Figure 9: 22 – 28 kHz USV acoustic characteristics of HAD-1 vs. LAD-1 rats.



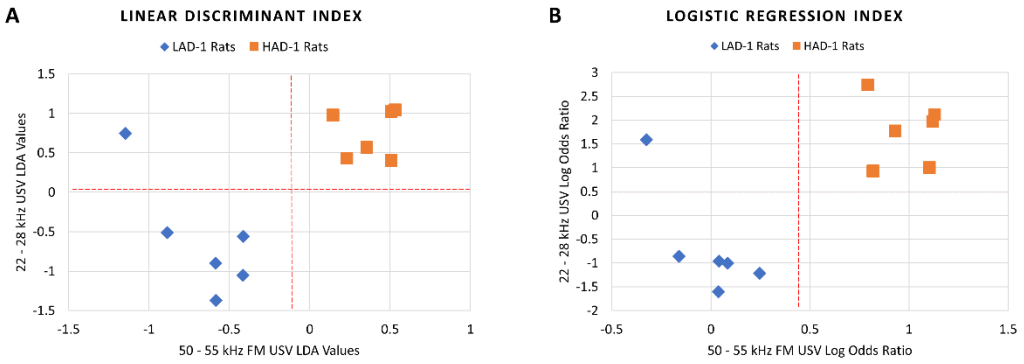
Linear mixed models were used to assess the effect of selective breeding (HAD-1 vs. LAD-1) on the acoustic characteristics of spontaneously emitted 22 – 28 kHz USVs. A) Mean Frequency of individual calls did not differ between HAD-1 and LAD-1 rats ($p = 0.303$). B) Duration of the calls emitted by LAD-1 rats was significantly higher than those emitted by HAD-1 rats ($p < 0.0001$). C) Bandwidth of calls did not differ between HAD-1 and LAD-1 rats ($p = 0.151$). D) Power of each call was significantly different between HAD-1 and LAD-1 rats ($p<0.0001$), though no clear direction of this effect was observed.

Figure 10: 50 – 55 kHz FM USV acoustic characteristics of HAD-1 vs. LAD-1 rats.



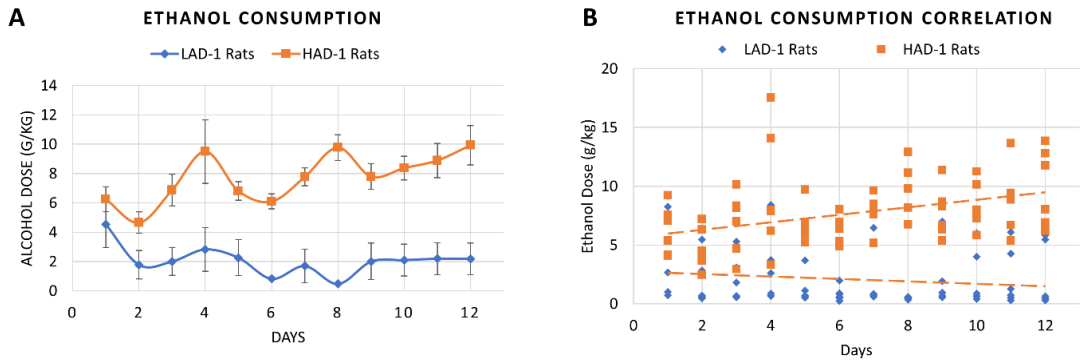
Linear mixed models were used to assess the effect of selective breeding (HAD-1 vs. LAD-1) on the acoustic characteristics of spontaneously emitted 50 – 55 kHz Frequency Modulated USVs. A) Mean Frequency of the calls emitted by HAD-1 rats was higher than those emitted by the LAD-1 rats ($p < 0.0001$). B) Duration of the calls emitted by HAD-1 rats was significantly higher than those emitted by LAD-1 rats ($p < 0.0001$). C) Bandwidth of calls made by LAD-1 rats was wider than those made by HAD-1 rats ($p < 0.0001$). D) Power of each call was significantly different between HAD-1 and LAD-1 rats ($p<0.01$), though once again no clear direction of this effect was observed.

Figure 11: Maximal separation between HAD-1 and LAD-1 rats achieved via LDA and Binomial Logistic Regression Analyses using 22 – 28 kHz and 50 – 55 kHz FM USV data.



A) Linear Discriminant Analysis provided accurate discrimination of 11/12 rats based on 22 – 28 kHz USV data and a complete discrimination of 12/12 rats based on 50 – 55 kHz FM USV data. Horizontal line represents the discrimination threshold for 22 – 28 kHz calls, vertical line represents the discrimination threshold for 50 – 55 kHz FM calls. B) Binomial Logistic Regression applied to the complete data set matched the maximal separation achieved by the LDA.

Figure 12: Total alcohol consumption during 4 weeks of 24-hour chronic intermittent ethanol availability sessions.



A) HAD-1 rats consumed significantly more alcohol than the LAD-1 rats ($p < 0.001$). B) Pearson's correlation analysis revealed an escalation in alcohol intake over time in HAD-1 rats ($r = 0.387$, $p < 0.001$), but not in LAD-1 rats ($r = -0.153$, $p = 0.20$).

Table 3: Coefficients for linear discriminant analysis.

COEFFICIENTS FOR LINEAR DISCRIMINANT ANALYSIS.

USV Subtype	$\beta_{\text{Mean Frequency}}$	β_{Duration}	$\beta_{\text{Bandwidth}}$	β_{Power}
22 – 28 kHz	-0.3676575	-0.8221113	0.4190684	-0.7509814
50 – 55 kHz FM	0.6065341	0.7826930	-0.2455201	-0.2847963

Note. The coefficients represent the β values associated with each acoustic characteristic used to calculate the linear discriminant values for each 22 – 28 kHz or 50 – 55 kHz frequency modulated (FM) call. The magnitude of these coefficients represents the contribution of the respective acoustic characteristic to the total separation achieved by the LDA model.

Table 4: Coefficients for binomial logistic regression.

COEFFICIENTS FOR BINOMIAL LOGISTIC REGRESSION.

USV Subtype	β_0	$\beta_{\text{Mean Frequency}}$	β_{Duration}	$\beta_{\text{Bandwidth}}$	β_{Power}
22 – 28 kHz	0.51509 ± 0.03061	-0.62074 ± 0.03114	-1.46758 ± 0.04180	0.94335 ± 0.04741	-1.17084 ± 0.03414
50 – 55 kHz FM	0.61281 ± 0.04622	0.51768 ± 0.04557	0.80332 ± 0.05774	-0.20861 ± 0.04334	-0.27239 ± 0.04560

Note. The coefficients represent the β values associated with the intercept (β_0) and each acoustic characteristic used to calculate the log odds ratio for each 22 – 28 kHz or 50 – 55 kHz frequency modulated (FM) call. The magnitude of these coefficients represents the contribution of the respective acoustic characteristic to the total separation achieved by the logistic regression model.

DISCUSSION

Ultrasonic vocalizations are established markers of positive and negative affective states in rodents. A plethora of studies have shown that different types of USV calls can be elicited by a wide variety of behavioral and pharmacological manipulations. These calls are especially sensitive to modulation by dopaminergic, as well as, cholinergic agonists and antagonists (Brudzynski, 1994; Brudzynski et al., 2011; Simola, 2015). In the present study, we explored whether USV acoustic characteristics from alcohol-naïve rats can be used to discriminate between selectively bred high- and low-alcohol drinking rats. We found clear differences in the acoustic characteristics of 50 – 55 kHz FM and 22 – 28 kHz USV calls between alcohol-naïve HAD-1 and LAD-1 rats. Moreover, we were able to use machine-learning algorithms to accurately identify rats as HAD-1 vs LAD-1 exclusively on the basis of USV acoustic parameter data.

Frequency modulated 50 – 55 kHz USVs may serve as biomarkers of activity in the mesolimbic dopaminergic system. This activity is associated with positive affective states (i.e., reward and positive reinforcement). Studies have shown that these calls can be directly evoked by dopamine release (Scardochio et al., 2015) and modulated by pharmacological manipulations of dopaminergic transmission. For instance, administration of psychostimulants such as cocaine, amphetamine, and methylphenidate, which are known to increase mesolimbic dopaminergic activity, dose dependently increases the total number of 50 – 55 kHz FM USV calls in rodents (Burgdorf et al., 2001; Ahrens et al., 2009; Maier et al., 2012). In addition to the increased call counts, amphetamine administration has also been shown to increase in the mean frequency and bandwidth of 50 – 55 kHz calls (Brudzynski et al., 2011; Simola, 2015). Furthermore, these changes could be reversed via D1 and D2 receptor antagonism, or through

experimental degradation of the nigrostriatal dopaminergic pathway (Wintink and Brudzynski, 2001; Ciucci et al., 2009; Wright et al., 2013).

In the present study, we showed that alcohol-naïve HAD-1 rats not only emitted more spontaneous 50 – 55 kHz FM USVs than the LAD-1 rats, but the calls emitted by the HAD-1 rats also had a higher mean frequency, narrower bandwidth, and longer duration than the calls emitted by the LAD-1 rats. These results, in combination with the previous findings about the neural substrates underlying 50 – 55 kHz USVs, suggest that HAD-1 rats may have enhanced basal dopaminergic activity as compared to the LAD-1 rats. Interestingly, alcohol-preferring HAD and P rat lines display 10 – 30% lower tissue levels of dopamine and its metabolites (dihydroxyphenylacetic acid and homovanillic acid) in the nucleus accumbens (Acb) and the anterior striatum when compared to their LAD and NP counterparts, respectively (Gongwer et al., 1989; Murphy et al., 2002). Though these results may seem paradoxical at first, lower in vitro basal tissue dopamine levels are thought to mediate increased dopaminergic activity as a compensatory mechanism. Indeed, ventral tegmental area (VTA) dopamine neurons were found to have increased burst firing in P rats (Morzorati and Marunde, 2006). Although a similar increase in burst firing was not seen in HAD-1 rats, other in vivo studies have shown increased levels of extracellular dopamine in the HAD-1 rats when compared with the LAD-1 rats (Katner and Weiss, 2001). This is consistent with increased dopaminergic activity. More recent research has shown that HAD-1 rats have elevated catechol-O-methyl transferase (COMT) mRNA in the posterior VTA, Acb shell, and central amygdala compared with LAD-1 rats (McBride et al., 2012, 2013b). COMT enzymatically breaks down dopamine, and other catecholamines, which provides further support for increased dopaminergic activity in the extended amygdala of HAD-1 vs LAD-1 rats. Although we did not directly measure dopaminergic activity in the present

study, known differences in dopaminergic transmission between HAD-1 and LAD-1 rats are consistent with our findings that these rat lines can be identified exclusively according to the acoustic characteristics of their emitted 50 – 55 kHz FM USVs.

Emission of 22 – 28 kHz USVs, on the other hand, is associated with medial cholinergic transmission. Contrary to 50 - 55 kHz FM USVs, 22 - 28 kHz USVs are associated with anxiety and other negative affective states (Brudzynski, 2009, 2013). These types of calls can be directly induced with cholinergic activation of the medial hypothalamic/preoptic region in rodents, via carbachol (Brudzynski and Bihari, 1990), and conversely, can be antagonized with application of cholinergic antagonists, such as atropine and scopolamine (Brudzynski, 2001). Furthermore, the acoustic characteristics, such as call duration, power, and bandwidth, of 22 – 28 kHz USVs were also modulated by carbachol administration in a dose dependent manner (Brudzynski, 1994).

In this study, we found that the significant differences in the acoustic characteristics of spontaneously emitted 22 – 28 kHz USV call duration and power between HAD-1 and LAD-1 rats varied enough to develop a linear discriminant model that could discriminate between HAD-1 and LAD-1 rats with high accuracy. Unfortunately, no study to date has explored potential differences in cholinergic transmission between HAD-1 and LAD-1 rats. Therefore, future studies will need to investigate the cholinergic system in HAD-1 and LAD-1 rats, in order to determine whether the differences in 22 – 28 kHz USV acoustic characteristics between HAD-1 and LAD-1 rats are associated with corresponding differences in cholinergic transmission in these rat lines.

Previous work conducted in our lab showed that 22 – 28 kHz USV acoustic features could be used to accurately discriminate between calls emitted by P vs NP rats (Reno et al., 2017). Moreover, these differences were in line with published literature on

differences in cholinergic transmission between P and NP rats (Bell et al., 2016). Here we show that similar to the P/NP rat lines, alcohol-naïve HAD-1 and LAD-1 rats also have differences in the acoustic characteristics of spontaneously emitted 22 – 28 kHz USVs. However, as indicated above, unlike the P/NP rats, HAD-1 and LAD-1 rats also have measurable differences in the acoustic characteristics of spontaneous 50 – 55 kHz FM USVs.

Finally, we show that acoustic characteristics of 22 – 28 kHz and 50 – 55 kHz FM USVs spontaneously emitted by alcohol-naïve HAD-1 and LAD-1 rats correlate with future alcohol consumption of these rats. Our current findings provide novel evidence that USV acoustic characteristics can be used to discriminate between alcohol-naïve HAD-1 and LAD-1 rats, and may serve as biomarkers in rodents with a predisposition for, or against, excessive alcohol intake.

Chapter 5: Rodent Ultrasonic Vocalizations as Biomarkers of Future Alcohol Use: A Predictive Analytic Approach [5]

ABSTRACT

Excessive alcohol consumption has a vast, negative impact on society. Rodent models have been successful in furthering our understanding of the biological underpinnings that drive alcohol consumption. Rodents emit ultrasonic vocalizations (USVs) that are each composed of several acoustic characteristics (e.g., frequency, duration, bandwidth and power). USVs reflect neurotransmitter activity in the ascending limb of the mesolimbic dopaminergic and cholinergic neurotransmitter systems and serve as non-invasive, real-time biomarkers of dopaminergic and cholinergic neurotransmission in the limbic system. In the present study, we recorded spontaneously emitted USVs from alcohol-naïve Long-Evans (LE) rats and then measured their alcohol intake. We compared the USV acoustic characteristics and alcohol consumption data from these LE rats with previously published data from selectively bred high- (P and HAD-1) and low-alcohol (NP and LAD-1) drinking lines from studies with the same experimental method. Predictive analytic techniques were applied simultaneously to this combined data set and revealed that: (a) USVs emitted by alcohol-naïve rats accurately discriminated among high-alcohol consuming, LE, and low-alcohol consuming rat lines, and (b) future alcohol consumption in these same rat lines was reliably predicted from the USV data collected in an alcohol-naïve state. To our knowledge this is the first study to show that alcohol consumption is predicted directly from USV profiles of alcohol-naïve rats. Because USV acoustic characteristics are sensitive to underlying neural activity, these findings suggest

[5] This is a post-peer-review, pre-copyedit version of an article published in Cognitive, Affective, and Behavioral Neuroscience. The final authenticated version will be soon available online (12/2017). NM was responsible for experiment design, data collection, analysis, interpretation, and writing of this manuscript.

that baseline differences in mesolimbic cholinergic and dopaminergic tone could determine the propensity for future alcohol consumption in rodents.

INTRODUCTION

Excessive alcohol consumption has a vast societal impact with far-reaching consequences for drinkers and the people around them. In the United States, the cost of alcohol misuse from lost workplace productivity, healthcare expenses, criminal justice expense, and motor vehicle crashes was estimated to be $249.0 billion (Sacks et al., 2015). Due to the vast cost associated with alcohol abuse, a variety of approaches have been developed to identify the biological underpinnings that drive alcohol consumption.

One such approach in preclinical research is the development of selectively-bred rat lines that voluntarily demonstrate excessive alcohol consumption (Murphy et al., 2002). Examples of such lines include alcohol preferring (P) rats and high alcohol drinking (HAD-1) rats, as well as their counterparts the alcohol non-preferring (NP) rats and low alcohol-drinking (LAD-1) rats. These lines are widely used by laboratories around the world and fit many of the criteria for an animal model of alcohol use disorders (AUDs) (Rodd et al., 2004; Bell et al., 2016). Research on these lines has led to the identification of therapeutic targets that are also effective in non-selective outbred strains, such as the Long-Evans and Wistar rats (Bell et al., 2012, 2016; McBride et al., 2014).

Converging lines of evidence from the selectively-bred rat lines and human literature suggest that genetic variation in dopaminergic and cholinergic neurotransmitter systems is associated with alcohol use and abuse (Stewart and Li, 1997; Enoch and Goldman, 2001; Morzorati and Marunde, 2006; McBride et al., 2013b; Tawa et al., 2016). One approach to studying the role of cholinergic and dopaminergic

neurotransmission in AUDs is to use biomarkers that are associated with activity in these neurotransmitter systems.

Ultrasonic vocalizations (USVs) are an established, non-invasive, real-time, functional biomarker of dopaminergic and cholinergic transmission in rodent limbic system (Brudzynski, 2015). USVs are separated into two common subtypes: 50 – 55 kHz frequency modulated (FM) USVs, which are emitted in response to rewarding stimuli and reflect positive affective states (Burgdorf and Panksepp, 2006; Ahrens et al., 2009; Ciucci et al., 2009; Maier et al., 2012; Buck et al., 2014b; Popik et al., 2014; Avvisati et al., 2016; Brenes et al., 2016; Roccaro-Waldmeyer et al., 2016; Williams and Undieh, 2016) and 22 – 28 kHz USVs, which are emitted in response to aversive stimuli and reflect negative affect (Brudzynski et al., 1993; Lindquist et al., 2004; Wang et al., 2008; Bardin et al., 2010; Chen et al., 2012; Parsana et al., 2012; Berger et al., 2013; Furlanetti et al., 2015).

As real-time, functional biomarkers of activity in the mesolimbic dopaminergic and cholinergic neurotransmitter systems (Wöhr and Schwarting, 2013; Brudzynski, 2015), USVs can serve as important tools for understanding the role of these systems in AUDs as well as a number of other neurological disorders (Ciucci et al., 2009; Scattoni et al., 2009; Basken et al., 2012; Machold, 2013; Pan et al., 2014; Brenes et al., 2016; Steele et al., 2017). However, due to the time- and labor-intensive nature of USV analyses, this methodology is vastly underutilized. In fact, nearly all published USV studies report only short durations of USV activity (e.g., less than 30 minutes) in counts alone (Armstrong et al., 2001; Burgdorf et al., 2007; Portfors, 2007; Scattoni et al., 2009; Brudzynski, 2015; Johnson et al., 2015; Kaniuga et al., 2016), but do not provide a full USV profile that could be revealed by repeated, long-duration assessment periods. Our development of WAAVES, a highly accurate algorithm that automates the analysis of

USVs (Reno et al., 2013; Reno and Duvauchelle, 2014) has allowed us to analyze data from experiments with relatively long timelines, something that was previously not feasible using manual techniques. In addition, WAAVES also allows an examination of each USV in much greater detail. Specifically, in addition to counting the number of USVs, the WAAVES analyses quantify several acoustic characteristics associated with each USV. These characteristics include the USV mean frequency in kilohertz, duration in milliseconds, bandwidth in kilohertz, and relative power in decibel. The result is a rich, multi-dimensional representation of each unique USV. These characteristics can be combined into large data sets for analyses using advanced statistical models and predictive analytics to examine the intricate relationships between rat USV characteristics and behavioral phenotypes, such as alcohol seeking and consumption. This is the approach taken in some of our recent work (Mittal et al., 2017b; Reno et al., 2017), and the current report.

OVERVIEW OF THE PRESENT STUDY

The overriding aim of the present work is to provide the first empirical test of the hypothesis that rodent ultrasonic vocalizations emitted by alcohol-naïve rats can be used to predict their future alcohol consumption. To test this hypothesis, we conducted a new study using Long-Evans rats and merged these results with our published data obtained from selectively bred high- (P and HAD-1) and low-alcohol (NP and LAD-1) drinking lines. The result is an integrative analysis of USV data collected from five different rat lines that highlight a novel and powerful role of USVs as direct predictors of excessive alcohol consumption and biomarkers of neurotransmission in these rat lines.

Instead of focusing on a single strain of rat, in the present study, we take the more challenging, but more broadly informative approach of testing our hypothesis across a

number of related lines of rats, including selectively bred and non-selectively bred lines. With this goal in mind, we conducted a new experiment in Long Evans (LE) rats using the same procedures used in two previous studies in our lab, one conducted using P vs. NP rats (Reno et al., 2017) and the other using HAD-1 vs. LAD-1 rats (Mittal et al., 2017b).

The rest of this manuscript is organized as follows. In the next section, we introduce the new study using Long-Evans rats. This is followed by a description of the predictive analytic approach and results from the combined sample that includes the new Long-Evans data and the P/NP (Reno et al., 2017) and HAD-1/LAD-1 (Mittal et al., 2017b) studies. We conclude with a summary and integration of these results with the existing literature.

METHODS

Animals

Sixteen male Long-Evans rats were ordered from Harlan (Harlan Laboratories, Indianapolis, Indiana) at approximately 4 weeks of age. The average weight of the rats upon arrival was ~180 grams. Rats were handled 20 minutes per day, 5 days per week for 3 weeks to habituate them to the experimenters. The animals were housed in a 12-hour reverse-light cycle with lights out from 10:00 AM – 10:00 PM. All USV recording sessions and the subsequent EtOH availability sessions began at the beginning of the dark cycle at 10:00 AM. Rats had *ad libitum* access to food and water throughout all experiments. Data from the previously published studies consists of six HAD-1, six LAD-1 (Mittal et al., 2017b), ten P and four NP rats (Reno et al., 2017). All experiments were conducted in accordance with *Institutional Animal Care and Use Committee* guidelines.

Ultrasonic Vocalization Recordings

USVs were recorded from alcohol-naïve rats in 4-hour sessions for 3 days/week (e.g., Monday, Wednesday, Friday) for 2 weeks. CM16 microphones were used with an UltraSound Gate interface (Avisoft Bioacoustics) to record USVs at a 250-kHz sampling rate and a 16-bit resolution. The recording sessions started at the beginning of the dark cycle. The animals were weighed and transported to the recording room, where they were transferred into recording cages, which were identical to the home cages, but used only for the recording sessions. Since novelty or conspecific scents could affect rodent behaviors (Wöhr et al., 2008), each animal was assigned its own recording cage. The animals were given access to food and water in the cage. Based on rat and chamber size, we approximate the distance between the animal's head and the centered microphone to range from 5 cm to 28.4 cm. Following the recording sessions, the animals were transported back to the vivarium.

Analysis of USVs

USV recordings were analyzed using the WAAVES program developed in our laboratory (Reno et al., 2013; Reno and Duvauchelle, 2014). This program reads USV containing audio files and produces a frequency spectrogram, which is subsequently analyzed using MATLAB's *Image Processing Toolbox* (MathWorks, Inc., Natick, MA). The program utilizes a variety of filters to separate USV calls from background noise, and several measurements of interest are quantified and extracted from each call. For 50 – 55 kHz FM USVs, WAAVES identifies sound objects with a minimum duration of 5 ms occurring in a range of 30 – 120 kHz. An inter-call interval of 10 ms was used to discriminate between individual calls and avoid counting call fragments as separate calls. FM USVs were defined as calls that varied more than 5 kHz over the entire duration of the call. 22 – 28 kHz calls were identified as sound objects occurring in a frequency

range of 20 to 30 kHz with a minimum duration of 200 ms. An inter-call interval of 100 ms was used to separate individual calls. Once the calls are identified, several measurements of interest are extracted from each USV call and stored for subsequent analysis. In the present study, the mean frequency, duration, bandwidth, and power for 50 – 55 kHz FM and 22 – 28 kHz USV calls were extracted for statistical analysis.

Validation Process for WAAVES Automation

Validation of WAAVES-generated USV data requires correspondence with human-derived analyses. Experimenters manually analyzed subsets of USV data recorded during the experiment to compare human assessment with WAAVES output. A subset of 10-min USV files recorded from LE rats (22 – 28 kHz calls: 40 files; 50 – 55 kHz calls: 40 files) were used for manual validation. The total number of calls identified via manual analysis was correlated with the total number of calls identified by the automated WAAVES program. The correlation coefficients are reported in the results.

Ethanol Availability Sessions

Following the USV recording rats were given access to 24-hour chronic intermittent ethanol sessions 3 days/week for 4 weeks as previously described (Mittal et al., 2017b; Reno et al., 2017). The sessions began at the beginning of the dark cycle. The rats were weighed at the beginning of each session and received 3 bottles containing either water, 15% EtOH, or 30% EtOH. The bottles were removed 24 hours later and weighed to measure ethanol or water consumption. Any potential loss of fluids due to spillage was insignificant, and therefore not accounted for in the present study. The dose of EtOH in g/kg was measured using the following formula:

*EtOH Dose (g/kg) = ((0.3*Change in weight of 30% bottle) + (0.15* Change in weight of 15% bottle)) * 0.8 * (1 / (0.001* Weight of rat in grams))*

Advanced Statistical Methods for Testing the USV – Alcohol Consumption Relationship

Traditional USV studies have focused primarily on differences in total call counts in the 22 – 28 kHz and 50 – 55 kHz FM categories, across experimental groups. This approach has proven to be a reliable index of underlying neurotransmission and is sensitive to experimental and pharmacological manipulations (Knutson et al., 2002; Ciucci et al., 2009; Brudzynski, 2013; Pan et al., 2014). Using this approach our lab has shown that USVs are responsive to a wide range of drugs of abuse and can serve as a useful model for investigating the neural basis of drug reward and the development of drug craving, behavioral sensitization and drug reward tolerance (Ahrens et al., 2009, 2013; Ma et al., 2010; Maier et al., 2012; Reno et al., 2015; Thakore et al., 2016). Many of these studies, including those from our lab, have used standard parametric statistical approaches (e.g. repeated-measures or two-way ANOVAs) to analyze the USV count data. However, these methods are not sufficiently powerful to analyze the USV acoustic characteristic data captured with WAAVES.

Predictive analytic approaches, such as linear discriminant analysis (LDA), are required when the goal is to determine whether a linear combination of the USV acoustic characteristics can successfully discriminate between rat lines (e.g. P vs. NP or HAD-1 vs. LAD-1 rats). Using LDA on the USV data produces a linear equation that takes the following form:

$$LDA\ score = \beta_{Mean\ Frequency} * Mean\ Frequency + \beta_{Duration} * Duration + \beta_{Bandwidth} * Bandwidth + \beta_{Power} * Power$$

This method computes the β coefficients that can be used to combine the data from all four acoustic characteristics into a singular 'LDA score' that maximizes the percent of USV calls classified into the appropriate experimental classes (e.g., P vs. NP

or HAD-1 vs. LAD-1 rats). For example, if we have a dataset consisting of 22 – 28 kHz calls made by P and NP rats, then the LDA method will produce β coefficients that maximize the difference between the LDA scores of the P and the NP rats respectively, such that we can take the average LDA scores of each group and construct a decision threshold halfway between the two averages to make predictions about unclassified data. That is, if the LDA score (calculated using the equation above) of an unknown animal falls below the decision threshold, then we predict that the rat is a P rat and if it is above the threshold then we predict that the rat is an NP rat (or vice-versa). An additional advantage of this method is that we are able to reduce the four USV characteristics into a one-dimensional LDA score, which can be easily plotted. The LDA method can be implemented using the freely available R package "MASS" (Venables and Ripley, 2002).

RESULTS

Using LDA to Accurately Separate Selectively-bred High- and Low-drinking Rat Lines

Our lab has successfully used linear discriminant analysis to classify HAD-1 and LAD-1 rats with 100% accuracy solely on the basis of the 50 – 55 kHz FM USV data (Mittal et al., 2017b). Similarly, we have also been able to use 22 – 28 kHz USV data to discriminate alcohol-naïve HAD-1 rats from LAD-1 rats with a classification accuracy of 81.96% (Mittal et al., 2017b) and alcohol-naïve P rats from NP rats with 100% accuracy (Reno et al., 2017). Our work on the differences in USV characteristics between the HAD-1/LAD-1 and the P/NP lines suggests that traits associated with alcohol consumption in these lines may also affect USV production. Consequently, USVs may serve as a potential biomarker for excessive alcohol consumption. However, it is also possible that the differences in USV characteristics between these lines may simply have

been a byproduct of genetic drift stemming from potential inbreeding within each selectively-bred line (Crabbe et al., 1990). The P and NP rats were derived from a closed colony of Wistar rats, while the HAD-1 and LAD-1 rats were developed using the N/Nih heterogeneous stock (Gongwer et al., 1989; Bell et al., 2012). Therefore, it is unlikely that the similar differences in USV characteristics observed across these different high and low alcohol consuming lines, derived from distinct breeder stocks, are simply a result of random genetic drift during the selective-breeding process.

Using LDA to Predict Alcohol Consumption in a Combined HAD-1, LE, LAD-1 Sample

Long-Evans rats, derived from mating a Wistar female with a wild grey rat (Weisbroth, 1969), are commonly used by many labs to study alcohol use and abuse. Although derived from a different breeder stock, HAD-1 and LAD-1 rats show stratification in alcohol consumption levels. Therefore, we first examined alcohol consumption in the combined sample as shown in Figure 1A. We saw that while the HAD-1 rats consume high levels of alcohol and the LAD-1 rats consumed low levels of alcohol, the LE rats show moderate levels of consumption ranging from HAD-1 levels to LAD-1 levels (Figure 1A). Second, we assessed whether similar stratification could be observed in the USV data amongst the HAD-1, LAD-1, and LE rat lines. All of the USVs emitted during the two-week period were used for this analysis without any references to recording days. Using LDA, we estimated the linear discriminant scores for the three rat lines. When the LDA method is used to discriminate between two classes of data a single linear equation is produced to generate a decision threshold that separates the two classes. However, when dealing with three classes, two decision thresholds are needed. Therefore, the LDA method produced two linear discriminant scores for each of the 50 – 55 kHz FM (Figure 2A) and 22 – 28 kHz USV (Figure 2B) categories.

We found that the linear discriminant scores from 50 – 55 kHz FM USVs showed some degree of separation between the HAD-1 and LE rats (90.91% separation), and the HAD-1 and LAD-1 rats (100% separation; Figure 2A), though no clear distinction existed between the LDA scores from the LE and LAD-1 rats (54.54% separation). On the other hand, the LDA scores from the 22 – 28 kHz USV data showed clear clustering across the HAD-1, LAD-1 and LE rat lines (HAD-1 vs. LE separation: 100%; LAD-1 vs. LE separation: 95.45%; HAD-1 vs. LAD-1 separation: 100%; Figure 2B). Because 50 – 55 kHz FM and 22 – 28 kHz USV emissions are mediated through the mesolimbic dopaminergic and cholinergic neural pathways (Wöhr and Schwarting, 2013; Brudzynski, 2015), these results suggest that basal differences in these transmitter systems may exist between the selectively-bred HAD/LAD and the outbred LE rat lines. However, as no study to date has directly explored these differences across these three rat lines, this hypothesis requires further work for serious consideration.

Linear Discriminant Scores Predict Future Alcohol Consumption:

Equipped with the linear discriminant scores based on the USV data from alcohol-naïve rats and the future alcohol consumption data, we tested the hypothesis that alcohol consumption levels in each rat can be predicted from their USV acoustic characteristics. To test this hypothesis, we generated a multivariate regression model that used the four linear discriminant values described above (i.e., from Figures 2A and 2B) to retrospectively predict total alcohol consumption for each rat during the subsequent alcohol availability sessions. Multivariate linear regression modeling strongly supported our hypothesis and revealed that the linear discriminant scores could be used to predict future alcohol consumption in these rat lines (multiple r squared = 0.7532, $F_{4,23} = 17.55$, $p < 0.0001$). Critically, to ensure that this linear model did not "over fit" the data, we used

the ‘leave-one-out’ cross-validation method. This is a commonly used validation technique that yields a conservative estimate of the accuracy of the regression model (Kearns and Ron, 1999). Using this method, for every rat a model is trained excluding that rat and the model is then used to make predictions on the data from the excluded rat. Thus, the model predicted values are generated on ‘unseen’ data that are not used in building the model. As shown in Figure 3A, the model predicted consumption values showed a high degree of correlation with the total alcohol consumption of the HAD-1, LAD-1 and LE rats during the four-week alcohol access period (r squared = 0.5999, $F_{1,26}$ = 38.99, $p < 0.0001$; Figure 3A). This finding provides the first direct evidence that USV acoustic characteristics can predict future alcohol consumption in alcohol-naïve rats. Importantly, this finding is not focused on a single strain of rats, but rather holds across three strains that are commonly used in alcohol research. The breadth of this finding across strains attests to its generality.

One might argue that by including selectively bred high- and low-alcohol consuming lines in the multivariate regression we increased the likelihood of finding that USVs predict future alcohol consumption. This reasoning is problematic because it assumes that USV profiles differ across these lines in a way that is systematically related to alcohol consumption. This is an empirical question that is tested in the current report. Even so, we conducted a secondary analysis focused just on the LE rats. In this LE sub-sample, we continue to find that alcohol consumption is strongly predicted from the USV profiles ($F_{1,14}$ = 12.90, $p < 0.01$).

Using LDA to Predict Alcohol Consumption in a Combined P, LE, NP Sample

Given the shared ancestry between the LE and the P/NP rat lines, we also sought to investigate potential similarities or differences in USV acoustic characteristics amongst

these three lines. As shown in Figure 1B, the alcohol consumption of LE rats also ranges from NP rat consumption levels to P rat consumption levels. Therefore, we also tested whether linear discriminant scores could be used to accurately separate the LE rats from the P and the NP rats. As seen in Figure 2A, the linear discriminant scores from the 50 – 55 kHz FM USV data did not show any clear distinction between the three rat lines (P vs. LE separation: 80.77%; NP vs. LE separation: 65.00%; P vs. NP separation 71.43%). However, the 22 – 28 kHz USV associated LDA values showed clear clustering across the P, NP and LE rats (P vs. LE separation: 92.31%; NP vs. LE separation: 95.00%; P vs. NP separation 100%; Figure 2B). Since 22 – 28 kHz USVs are associated with tegmental cholinergic transmission, these results suggest that there may be differences in basal cholinergic activity within the limbic system across these three lines. No study has directly compared these neurotransmitter systems between P and LE rats. Together with the HAD/LAD results, these findings highlight a need for future studies to directly explore these systems and compare neurotransmitter activity across these rat lines.

Linear Discriminant Scores Predict Future Alcohol Consumption:

Next, we tested whether the linear discriminant scores based on the USV data from alcohol-naïve rats could also predict alcohol consumption in these rats[6]. Multivariate linear regression models showed that these LDA score could also be used to predict alcohol consumption in these rats (multiple r squared = 0.6995, $F_{4,25} = 14.55$, $p <$

[6] The USV data for the P/NP rats used in this study were collected from the control group of the P/NP study (Reno et al., 2017). Thus, these rats did not receive alcohol following the USV recording experiment making it difficult to assess the ability of USV data to predict future alcohol consumption in these rats. In order to overcome this problem, we used a random sampling method, whereby each of the P and NP rats used in this study were randomly assigned the alcohol consumption value of a P or NP rat from the alcohol-experience group of the P/NP study (Reno et al., 2017) and a linear model was generated to predict the alcohol consumption values of these rats based on the USV data. This process was repeated 10,000 times and the average prediction values from the 10,000 iterations were used to generate the reported correlation coefficients.

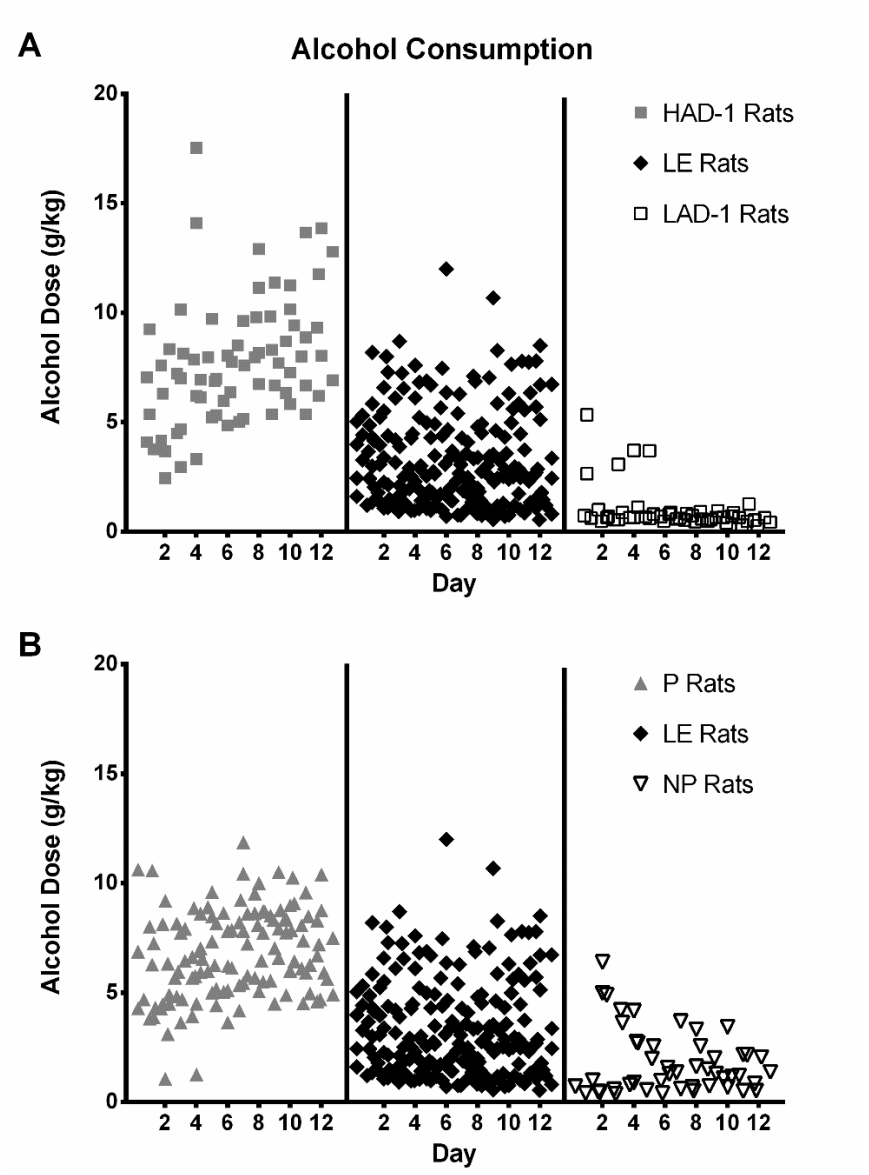
0.0001). The model predicted consumption values (generated using the leave-one-out cross-validation method as described above) showed a significant correlation with the alcohol consumption of the P, NP and LE rats (r squared = 0.5112, $F_{1,28} = 29.29$, $p < 0.0001$). Figure 3B provides a representation of this relationship using the assigned and predicted alcohol consumption values from one of the 10,000 simulations. These findings complement the HAD-LAD-LE data described above and provide strong evidence that USV data from selectively bred lines can be generalized to Long-Evans rats. Together these results provide clear and direct evidence that USV data can be used to predict alcohol consumption in rats.

As with the HAD/LAD analysis, one might argue that by including selectively bred high- and low-alcohol consuming lines in the multivariate regression, we increased the likelihood of finding that USVs predict future alcohol consumption. To address this potential concern, we conducted a secondary analysis focused just on the LE rats. In this LE sub-sample, we continue to find that alcohol consumption is strongly predicted from the USV profiles ($F_{1,14} = 30.77$, $p < 0.0001$).

Validation of USV Analysis

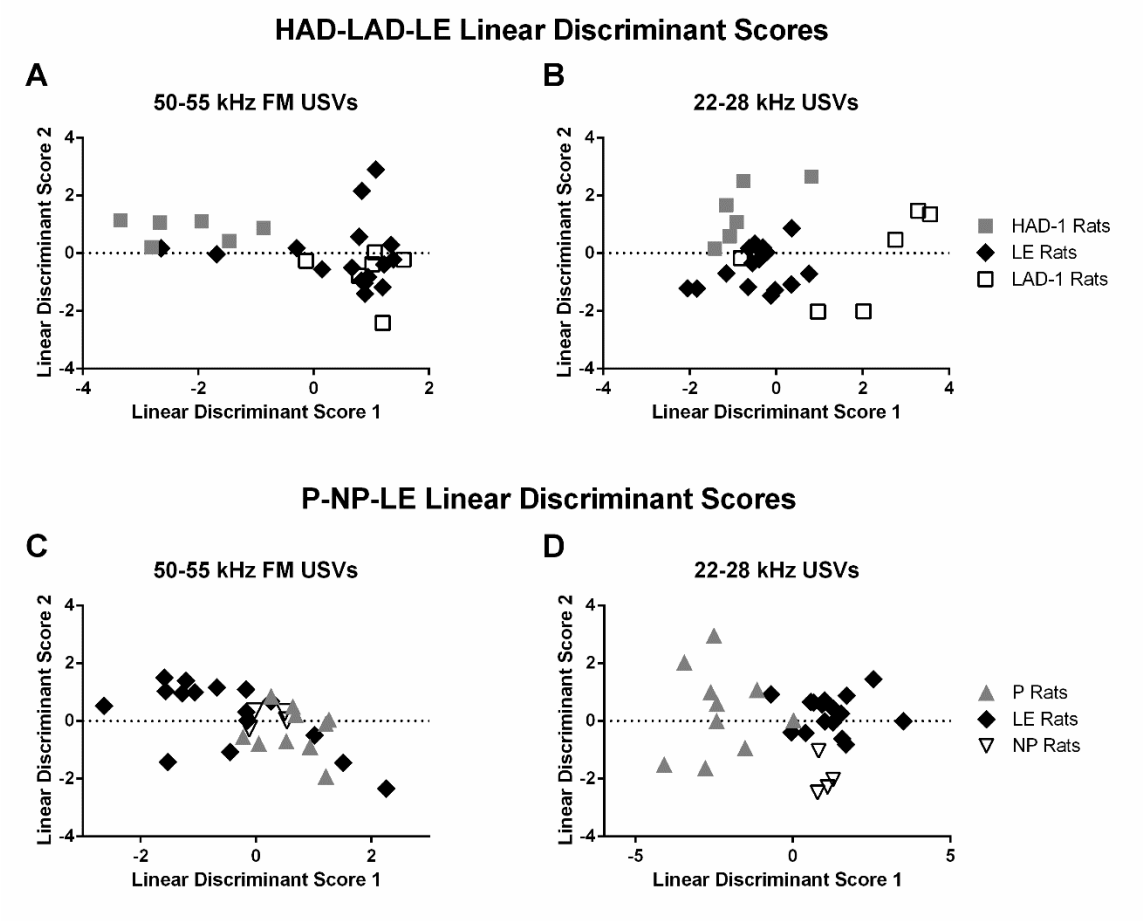
A subset of USV files was manually analyzed using Avisoft SASLab Lite (Avisoft Bioacoustics) to validate WAAVES output. WAAVES-automated analysis and manual analysis results were highly correlated for both the 22 – 28 kHz ($r = 0.996$; $p < 0.0001$) and 50 – 55 kHz calls ($r = 0.970$; $p < 0.0001$).

Figure 13: Daily alcohol consumption of HAD-1 – LE – LAD-1 and P – LE – NP rats across 12 drinking sessions.



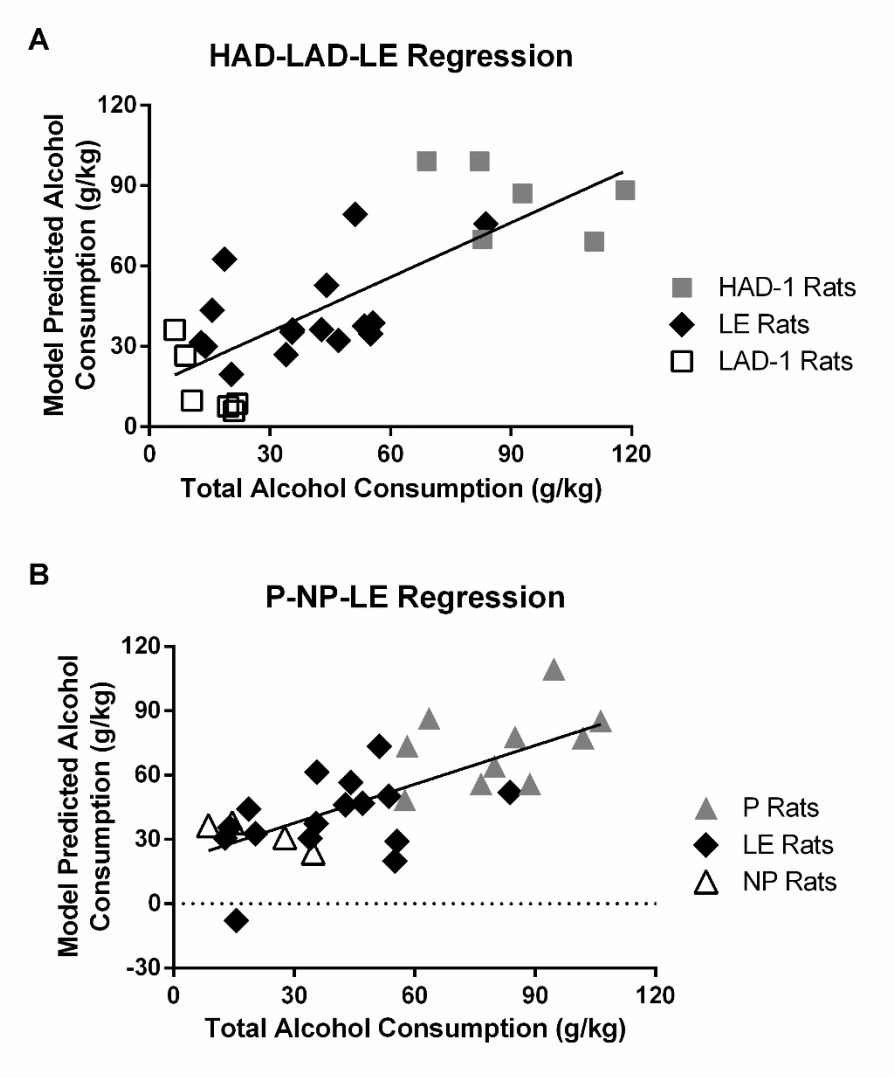
Individual symbols represent individual rats on each of the 12 EtOH sessions, with a slight offset on the x-axis used in order to minimize overlap between symbols. A) Daily alcohol consumption ranged from 2.45 – 17.53 g/kg for HAD-1 rats, from 0.55 – 12.00 g/kg for LE rats and from 0.28 – 5.34 g/kg for LAD-1 rats. B) Daily alcohol consumption ranged from 1.06 – 11.86 g/kg for P rats, from 0.55 – 12.00 g/kg for LE rats and from 0.36 – 6.39 g/kg for NP rats.

Figure 14: Maximal separation between HAD-1 – LE – LAD-1 and P – LE – NP rats achieved via Linear Discriminant Analysis using 22 – 28 kHz and 50 – 55 kHz FM USV data.



A) Linear Discriminant Analysis provided 90.91% separation accuracy between HAD-1 and LE rats, and 100% separation accuracy between HAD-1 and LAD-1 rats based on 50 – 55 kHz FM USV data. No clear separation was observed between LE and LAD-1 rats. B) Linear Discriminant Analysis based on 22 – 28 kHz USV data Showed 100% separation between HAD-1 and LE rats, 95.45% separation between LAD-1 and LE rats, and 100% separation accuracy between HAD-1 and LAD-1 rats. C) Linear Discriminant Analysis based on 50 – 55 kHz FM USV data did not show any clear separation between the P, LE and NP rat lines. D) Linear Discriminant Analysis based on 22 – 28 kHz USV data Showed 92.31% separation between P and LE rats, 95.00% separation between NP and LE rats, and 100% separation accuracy between P and NP rats.

Figure 15: Comparison of total alcohol consumption and model predicted alcohol consumption of HAD-1 – LE – LAD-1 and P – LE – NP rats during 4 weeks of 24-hour alcohol availability sessions.



A) Multivariate linear regression models developed using linear discriminant scores from HAD-1, LE and LAD-1 rats showed a high degree of correlation between the model predicted alcohol consumption and the actual consumption of these rats (r squared = 0.5999, $F_{1,26} = 38.99$, $p < 0.0001$). B) Multivariate linear regression models developed using linear discriminant scores from P, LE and NP rats also showed a high degree of correlation between the model predicted alcohol consumption and the actual consumption of these rats (r squared = 0.5112, $F_{1,28} = 29.29$, $p < 0.0001$).

DISCUSSION

The present study provided the first known test of the hypothesis that rodent ultrasonic vocalizations obtained while animals are alcohol-naïve serve as predictors for future alcohol consumption. Instead of focusing on a single rat strain, we took the more challenging, but more general, approach of testing this hypothesis across five rat lines: P, NP, HAD-1, LAD-1, and Long-Evans. We report the results from a new study with Long-Evans rats that uses the same procedure used in two other studies conducted in our lab: one with P and NP rats (Reno et al., 2017), and the other with HAD-1 and LAD-1 rats (Mittal et al., 2017b). In all three studies, we recorded spontaneously emitted USVs while the rats were alcohol-naïve and followed this with free alcohol access sessions in which alcohol consumption was measured. We combined these three data sets and utilized predictive analytic techniques to test this hypothesis.

Here we show that differences in USV expression, which is thought to be mediated through mesolimbic dopaminergic and cholinergic neurotransmitter systems is linked to the propensity for alcohol consumption in selectively bred and non-selectively bred rat lines. We showed that USVs could serve as real-time functional markers of activity in these systems. Two major findings emerged. First, we showed that linear discriminant scores calculated using the USV data could be used to discriminate Long-Evans rats from rat lines selectively-bred for high or low levels of alcohol consumption. These results point to potential differences in neurotransmission across these five rat lines and highlight the need for direct investigation of these systems. Second, using multivariate linear regression we found that future alcohol consumption could be predicted from USV profiles obtained while animals were alcohol naïve. Thus, these scores provide information about basal neurotransmission in these rat lines, unencumbered by any potential disruptions from alcohol exposure and highlight a novel

and potentially invaluable use of USV data as a predictor of alcohol consumption in these five rat lines.

Both pairs of rat lines tested in this study are known to have basal differences in dopaminergic and cholinergic transmission. For instance, P and HAD-1 rats have lower tissue levels of DA and its metabolites (DOPAC and HVA) in the nucleus accumbens and the anterior striatum as compared to their NP and LAD-1 counterparts (Murphy et al., 1982, 1987; Gongwer et al., 1989). This may be due to a potential deficit in the VTA DA projections in these rat lines. Indeed P rats have reduced DA projections from the VTA to the nucleus accumbens (Zhou et al., 1995), as well as, lower levels of DA D_2 receptor binding sites (McBride et al., 1993), as compared to the NP rats. In order to compensate for the lower levels of DA, P rats show increased burst firing of VTA DA neurons, though a similar increase was not seen in the HAD rats (Morzorati, 1998; Morzorati and Marunde, 2006). Alcohol consumption results in reduced D_2 auto-receptor function (Engleman et al., 2000, 2003), and enhanced DA efflux (Weiss et al., 1993; Thielen et al., 2004) and reuptake (Sahr et al., 2004).

The potential differences in cholinergic transmission between the selectively bred rat lines remain largely unexplored. However, the few studies that have been conducted suggest that the accumbal cholinergic system may be more active in P rats than NP rats (Bell et al., 2016). For instance, P rats have lower expression levels of striatal nicotinic acetylcholine receptor than NP rats (Tizabi et al., 2001). Studies have also identified higher expression levels of genes encoding choline acetyltransferase, muscarininc acetylcholine receptor 3, and channels responsible for the uptake and vesicular transport of acetylcholine during synthesis in the nucleus accumbens shell of P rats as compared to the NP rats (McBride et al., 2013a, 2013b). Furthermore, administration of nicotinic antagonists or desensitizing agents can reduce alcohol consumption in P rats (Rezvani et

al., 2010; Srisontiyakul et al., 2016). Although we did not directly explore these systems in our study, these studies provide support to the notion that the USV differences across these lines are a reflection of underlying differences in these neurotransmitter systems.

Although USV recordings are non-invasive and USV recording experiments are relatively easy to set up, USVs have remained relatively underutilized as important preclinical tools for the investigation of neural substrates underlying alcohol and drug abuse, primarily due to the manually intensive nature of the USV analysis process. The development of WAAVES (Reno et al., 2013; Reno and Duvauchelle, 2014), and other automated USV detection methods (Barker et al., 2014; Barker and Johnson, 2017) can help these problems and should encourage more labs to integrate USV recordings to their respective batteries of behavioral assays. Widespread use of USVs can unlock the true potential of this behavioral metric as a non-invasive correlate of underlying neural activity.

CONCLUSION

This present study provides the first known test of the hypothesis that rodent ultrasonic vocalizations obtained while animals are alcohol-naïve serve as a biomarker for and thus predict future alcohol consumption. We find support for this hypothesis in a combined sample of five rat lines commonly used in alcohol research. These results highlight a novel role of USVs as biomarkers of neurotransmission and the propensity for high levels of alcohol consumption in rodents.

Chapter 6: Spontaneous ultrasonic vocalization transmission in adult, male Long-Evans rats is age-dependent and sensitive to EtOH modulation

ABSTRACT

Ultrasonic vocalizations (USVs) are well-established markers of motivational and emotional status. Recent work from our lab has provided novel evidence for a role of USVs in models of alcohol use. USV acoustic characteristics can be used to accurately between rat lines selectively bred for high (alcohol-preferring and high-alcohol-drinking) versus low (alcohol-non-preferring and low-alcohol-drinking) alcohol consumption. In the present study we sought to explore the effect of age and alcohol-exposure on spontaneously emitted 50 – 55 kHz frequency modulated (FM) and 22 – 28 kHz USVs in adult, male Long-Evans rats. With the hypothesis that spontaneous USV emission depends on age and can be influenced by alcohol exposure, we examined USV data collected during a 24-week experiment in male Long-Evans rats. Experimental phases included Baseline (2 wks), 24-hr EtOH Access (4 wks), 4-hr EtOH Access (2 wks), 2-hr EtOH Access (3 wks) and 1-hr EtOH Access (3 wks) followed by two cycles of Abstinence (2 wks and 4 wks) and Reinstatement (1 wk and 3 wks). Findings showed that adult, male Long-Evans rats emit spontaneous 22 – 28 kHz and 50 – 55 kHz FM USVs. Analyses of USV counts and acoustic characteristics (i.e. mean frequency, duration, bandwidth and power) revealed distinct age-dependent phenotypes in both 50 – 55 kHz FM and 22 – 28 kHz USV transmission that were modulated by ethanol exposure. These results highlight unique emotional phenotypes in male Long-Evans rats that are differentially modulated by alcohol experience.

INTRODUCTION

Researchers have long recognized that rats are a highly vocal species, that emit ultrasound calls to communicate environmental states with conspecifics (Nyby and Whitney, 1978; Brudzynski, 2015). Over the last two decades, studies have shown that different categories of ultrasonic vocalizations emitted by rodents are associated with distinct environmental and motivational states. Specifically, 22 – 28 kHz USVs are emitted in response to threatening and alarming situations (Brudzynski, 2001; Wöhr et al., 2005; Litvin et al., 2007), and 50 – 55 kHz frequency modulated (FM) USVs are emitted in response to rewarding stimuli (Panksepp and Burgdorf, 2000; Burgdorf et al., 2011; Simola et al., 2012). Moreover, emission of these USV subtypes is dependent on distinct neurotransmitter pathways: the 22 – 28 kHz calls are produced by activation of the medial cholinoceptive vocalization strip (Brudzynski, 2001; Kroes et al., 2007; Machold, 2013) and 50 – 55 kHz FM USVs are generated through the activation of the ascending mesolimbic system (Ciucci et al., 2009; Maier et al., 2012; Ahrens et al., 2013; Scardochio and Clarke, 2013; Johnson et al., 2015; Scardochio et al., 2015). Thus, spontaneous emissions of these USV subtypes (i.e. 22 – 28 kHz and 50 – 55 kHz FM calls) may serve as biomarkers of underlying neural activity in the cholinergic and dopaminergic system.

The dopaminergic and cholinergic systems also play an important role in the development of alcohol use disorders. For instance, alcohol is known to increase the efflux of dopamine into mesocorticolimbic regions (Weiss et al., 1993; Engleman et al., 2000), and administration of dopamine antagonists can reduce alcohol intake in rodents (Panocka et al., 1993; Hutchison et al., 2001). Moreover, variations in genes encoding catechol-o-methyltransferase (COMT), one of the enzymes responsible for the degradation of catecholamines, are associated with alcohol dependence (Tiihonen et al.,

1999; Voisey et al., 2011; Reilly et al., 2017). Cholinergic receptors in the ventral tegmental area (VTA) have been shown to regulate the reinforcing properties of ethanol (Ericson et al., 2003; Löf et al., 2007) and acute alcohol exposure can elevate muscarinic tone in the septohippocampal system (Ericson et al., 2010). Furthermore, cholinergic antagonist mecamylamine (Ericson et al., 1998) and partial agonist varenicline (Erwin and Slaton, 2014) have been shown to reduce alcohol consumption behaviors. Variations in genes encoding nicotinic (CHRNA) and muscarinic (CHRM2) cholinergic receptors have also been associated with subjective responses to alcohol and the risk for alcohol dependence (Wang et al., 2004, 2009; Ehringer et al., 2007; Joslyn et al., 2008; Jung et al., 2011). Together these results suggest that inter-individual variation in alcohol consumption levels may in part be modulated by basal activity in the cholinergic and dopaminergic system.

Since USVs are reflective of activity in these systems, it is possible that USV transmission may be sensitive to modulation by ethanol and specific USV patterns may be associated with alcohol consumption behaviors in rodents. Indeed, recent work from our lab has shown that acute and chronic alcohol exposure can alter the counts and acoustic characteristics of spontaneously emitted USVs in alcohol-preferring (P) and high-alcohol-drinking (HAD-1) rats (Reno et al., 2015; Thakore et al., 2016). We have also shown that spontaneous USVs emitted in an alcohol-naïve state can be used to discriminate between HAD-1 and low-alcohol-drinking (LAD-1), as well as, P and alcohol non-preferring (NP) rats with a high degree of accuracy (Mittal et al., 2017c; Reno et al., 2017). Furthermore, we have found that spontaneous USVs emitted by alcohol-naïve Long-Evans (LE) rats can be used to predict future alcohol consumption levels.

In the present study we extend our previous findings to explore the role of ethanol modulation on spontaneous USVs emitted by male LE rats. USVs were recorded from adult male LE rats for 4 hours per day, 3 days per week, over the course of 24 weeks, under a variety of ethanol availability conditions. We used multivariate regression and linear mixed models to assess the effect of time and ethanol modulation on total counts and acoustic characteristics of 50 – 55 kHz FM and 22 – 28 kHz USVs. Based on previous work in our lab and published literature, we hypothesized that USV counts and characteristics would be altered over time as the rats matured, and that these changes would be differentially modulated by ethanol exposure.

MATERIALS AND METHODS

Animals

Twenty-four male Long-Evans rats were ordered from Harlan (Harlan Laboratories, Indianapolis, Indiana) at approximately 4 weeks of age. Rats were handled 5 days per week for 3 weeks to habituate them to the experimenters before the beginning of any experiments. All experiments were conducted in accordance with *Institutional Animal Care and Use Committee* guidelines.

Ultrasonic Vocalization Recordings

USV recordings were conducted as described in previous studies (Mittal et al., 2017b). All rats were recorded under both alcohol-naïve and alcohol-available (or water-available for control rats) conditions. Following the habituation period, USVs were recorded for 4 hours and 10 minutes per day, 3 days/week over a 24-week experimental timeline (Figure 1). CM16 microphones were used with an UltraSound Gate interface (Avisoft Bioacoustics) to record USVs at a 250-kHz sampling rate and a 16-bit

resolution. On recording days, animals were weighed at the beginning of the dark cycle, transported to a test room, and placed into recording cages (which were identical to their home cage but only used for USV recordings). Recordings began immediately after the animals were placed in their respective cages. Following a 10-minute 'anticipatory' period, EtOH (or water) and food were introduced to the recording cages and USVs were recorded continuously for the next 4 hours. Each animal was assigned its own recording cage in order to prevent any non-specific behaviors related to novelty or conspecific scents (Wöhr et al., 2008). Based on rat and chamber size, we approximate the distance between the animal's head and the centered microphone to range from 5 cm to 28.4 cm. After the recording session, the animals were transported back to the vivarium and returned to their home cage.

Experimental Timeline

At the beginning of the dark cycle, animals were weighed and then transported from the vivarium to the behavioral testing room. Animals were pair-housed during the habituation period, but were separated and single-housed before beginning the experimental sessions. The experimental sessions were conducted 3-5 days per week (in the dark with only red illumination) and commenced for a total of 24 weeks. Figure 1 provides a schematic of the experimental timeline employed in this study. During the first two weeks (Baseline), all rats had access to three sipper tubes filled with water only. For the next phase (24-hr EtOH Access; 4 wks) the EtOH group had access to 3 sipper tubes (water, 15% and 30% EtOH) while the Control group had access to three water sipper tubes every other day (i.e. Monday, Wednesday, Friday). During this phase, animals had EtOH access during the entire 4-hr USV recording session and then received continued EtOH access in their home cage for the remaining 20-hrs. For the following phase (4-hr

EtOH Access; 2 wks) animals in the EtOH group had 4-hr access to ethanol every other day. During the next phase (2-hr EtOH Access; 3 wks) animals in the EtOH group had 2-hr access to ethanol every other day. After 2 hours the EtOH bottles were replaced with three bottles of water and USV recordings continued for 2 more hours. For the next phase (1-hr EtOH Access; 3 wks), the animals received 1-hour access to EtOH 5 days per week (i.e. Monday – Friday), but USV recording sessions were only conducted 3 days/week (i.e. Monday, Wednesday, Friday). During the recording sessions, EtOH bottles were replaced with water bottles after 1 hour and the recordings continued for 3 additional hours. Following the 1-hour sessions, the animals underwent 2 weeks of 'abstinence' where they did not receive any alcohol. USV recording sessions were not conducted during the abstinence period. After the 2-week abstinence period, animals underwent a 1-week 'reinstatement' period that was similar to the 1-hour EtOH access period (i.e. 1-hr EtOH access 5 days/wk, USV recordings 3 days/wk). Following the first reinstatement period the animals underwent a second abstinence period for 4 weeks. Animals did not receive any alcohol during this period and no USV recording sessions were conducted. For the final phase of the experiment, the animals underwent a second reinstatement period for 3 weeks, which was similar to the 1-hour EtOH access and the first reinstatement period. Fluid intake was assessed gravimetrically after each drinking interval.

WAAVES Automated Analysis of USVs

Ultrasonic vocalization recordings were analyzed using the WAAVES program (Reno et al., 2013; Reno and Duvauchelle, 2014). This program reads audio files and produces a frequency spectrogram. The spectrogram is then scanned for sound objects using MATLAB's Image Processing Toolbox (MathWorks, Inc. Natick, MA). For 50 –

55 kHz FM USVs, WAAVES identifies sound objects with a minimum duration of 5 ms occurring in a range of 30 – 120 kHz. An inter-call interval of 10 ms was used to discriminate between individual calls and avoid counting call fragments as separate calls. FM USVs were defined as calls that varied more than 5 kHz over the entire duration of the call. 22 – 28 kHz calls were identified as sound objects occurring in a frequency range of 20 to 30 kHz with a minimum duration of 200 ms. An inter-call interval of 100 ms was used to separate individual calls. These call parameters were derived from the existing literature as well as extensive trial-and-error testing in the laboratory. Once the calls are identified, several measurements of interest are extracted from each USV call and stored for subsequent analysis. The mean frequency, duration, bandwidth, and power for both 50-55 kHz FM and 22-28 kHz calls were used for statistical analysis.

Validation Process for WAAVES Automation

A subset of 10-min USV files recorded from LE rats (22 – 28 kHz calls: 40 files; 50 – 55 kHz calls: 40 files) during the experiment were manually analyzed and compared with WAAVES-generated assessments to ensure the accuracy of WAAVES outputs. The total number of calls identified via manual analysis was correlated with the total number of calls identified by the automated WAAVES program. The correlation coefficients are reported in the results.

Statistical Analyses of USV Counts and Acoustic Characteristics

Linear mixed models were used to analyze USV counts and acoustic characteristics as previously described (Mittal et al., 2017b; Reno et al., 2017). A standard statistical approach would utilize repeated measures ANOVA to analyze the USV data. In this approach, all calls emitted by a rat are used to calculate an average, and then any potential group differences in these averages are assessed. Thus, this method

results in loss of important information about the inter-individual variability in USV calls for each rat, which, in turn, reduces power. Linear mixed models allow us to overcome these problems by accounting for inter-individual variation while examining the effects of treatment (e.g. EtOH vs Control) and experiment stage on total USV counts and the patterns of USV acoustic characteristics (e.g. mean frequency, duration, bandwidth, or power).

Linear Mixed Models

We assessed differences in total USV counts and each of the four USV characteristics as a function of treatment or experimental stage using a linear mixed model (LMM) in R (R Core Team, 2015) using the package "lmerTest" (Kuznetsova et al., 2016). The linear models were generated to assess the effect of treatment, experimental phase, or an interaction of these factors for USV counts and each of the 4 acoustic characteristics of interest. Whenever a significant effect was observed a new reduced model was generated by removing the significant factor and compared with the original model using an ANOVA in order to assess the impact of the respective factor on the goodness-of-fit for the model. A random slope coefficient was included to protect against potential noise introduced by random day-to-day variation in call parameters for each rat.

Linear Regression

We used linear regression modeling to assess the effect of rat age on USV counts and acoustic characteristics. Since all subjects were age matched at the beginning of the experiment, the week of recording was used as a proxy for age.

RESULTS

The goal of this study was to investigate the effects of age and EtOH exposure on the counts and acoustic characteristics of spontaneously emitted 50 – 55 kHz FM and 22 – 28 kHz USVs in adult male Long-Evans rats. USVs were recorded 3 days/week, over 24 weeks under control (water only) or EtOH conditions (Figure 1). USV recording sessions consisted of a 10-minute anticipatory period during which animals were place in the recording chambers and recorded in the absence of food and water (or EtOH). Following the anticipatory period food and water (or EtOH) were added to the recording cages and USVs were continuously recorded for 4 hours.

Rate of USV Emissions

We began by examining the rate of 50 – 55 kHz FM and 22 – 28 kHz USVs emitted during the entire 24-week experiment. Since the anticipatory period is 10-minutes in duration, USV emission rate was measured in 10-minute bins. We found that the rate of calling for 50 – 55 kHz FM USVs emitted during the anticipatory period (Control: 7.715 ± 3.466; EtOH: 15.415 ± 7.181) was significantly higher than those emitted during the subsequent 4-hour recording session (Control: 0.291 ± 0.065; EtOH: 0.427 ± 0.090; $F_{1,22} = 4.616$, $p < 0.05$; Figure 2A). Moreover, while both EtOH and Control treated animals had a higher rate of 50 – 55 kHz FM USV emissions during the anticipatory period, only the EtOH treated animals showed a significant difference in the rate of emission ($t_{22} = 2.489$, $p < 0.05$). On the other hand, the 22 – 28 kHz USV emission rate was comparable between the anticipatory period (Control: 0.433 ± 0.151; EtOH: 0.576 ± 0.382) and the subsequent 4-hour recording session (Control: 0.555 ± 0.119; EtOH: 0.530 ± 0.048; Figure 2B).

Effect of Age on USV Emissions

Since the animals were approximately 2 months old at the time of the first recording session and approximately 8 months old by the time of the final recording session, we next sought to examine the effect of age on USV acoustic counts and characteristics. Since we were only interested in the effect of age, we focused solely on the water treated control animals for this analysis. We used linear regression to determine whether USV counts and acoustic characteristics changed as a function of time. USVs emitted during the anticipatory and the 4-hour recording periods were analyzed separately.

50 – 55 kHz FM USVs

We did not observe any significant relationship between time and weekly counts or acoustic characteristics of 50 – 55 kHz FM USVs emitted during the anticipatory period. On the other hand, we saw a significant reduction in 50 – 55 kHz FM USVs emissions over time during the 4-hour recording period ($F_{1,142} = 4.660$, $p < 0.05$; r-squared = 0.0318; Figure 3A). Moreover, there was also an increase in the duration ($F_{1,141} = 13.440$, $p < 0.001$; r-squared = 0.0870; Figure 3C) and a decrease in the bandwidth ($F_{1,141} = 19.440$, $p < 0.0001$; r-squared = 0.1210; Figure 3D) of these calls over time. No such effect of time was observed on the mean frequency (Figure 3B) or power (Figure 3E) of these calls.

22 – 28 kHz FM USVs

In line with the 50 – 55 kHz FM call data, we did not observe any significant relationship between time and weekly counts or acoustic characteristics of 22 – 28 kHz USVs emitted during the anticipatory period. Moreover, we also did not see any change over time in the total number of 22 – 28 kHz USVs emitted during the 4-hour recording

period (Figure 4A). However, there was a significant increase in the bandwidth of these calls over time ($F_{1,141} = 5.185$, $p < 0.05$; r-squared = 0.0355; Figure 4D). No such effect was seen in the mean frequency (Figure 4B), duration (Figure 4C) or power (Figure 4E) of these calls.

Ethanol Modulation of USV Emissions

Finally, we used linear mixed models to assess the effect of EtOH treatment on the counts and acoustic characteristics of spontaneously emitted 50 – 55 kHz FM and 22 – 28 kHz USVs. The calls emitted during the anticipatory period were analyzed separately from those emitted during the 4-hour recording session in the presence of water or EtOH.

50 – 55 kHz FM USVs

We did not observe any significant Treatment*Experiment Stage interaction, nor any main effect of treatment on the number of 50 – 55 kHz FM calls emitted during the anticipatory period (Figure 5A). However, there was a significant Treatment*Experiment Stage interaction for the mean frequency (Figure 5B) and power (Figure 5E), but not the duration (Figure 5C) or bandwidth (Figure 5D) of these calls. The significant interaction for mean frequency was observed in the linear ($t_{73} = 2.225$, $p < 0.05$) and the quadratic ($t_{1041} = 4.818$, $p < 0.0001$) terms of the model. Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model ($\chi^2 = 28.323$, $p < 0.0001$). The interaction for power was observed in the quadratic ($t_{936} = -3.318$, $p < 0.001$), cubic ($t_{6414} = 2.144$, $p < 0.05$) and the sixth order ($t_{16424} = 2.931$, $p < 0.01$) terms of the model. Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model ($\chi^2 = 24.261$, $p < 0.001$).

Similar to the anticipatory period results, we did not observe any significant Treatment*Experiment Stage interaction, nor any main effect of treatment on the number of 50 – 55 kHz FM calls emitted during the 4-hour recording period (Figure 6A). However, there was a significant Treatment*Experiment Stage interaction for the mean frequency (Figure 6B), bandwidth (Figure 6D) and power (Figure 6E), but not the duration (Figure 6C) of these calls. The significant interaction for mean frequency was observed in the cubic ($t_{7955} = 2.051$, $p < 0.05$) and the fifth order ($t_{11843} = -2.936$, $p < 0.01$) terms of the model. Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model ($\chi^2 = 17.369$, $p < 0.01$). The interaction for bandwidth was observed in the quadratic ($t_{4887} = 3.236$, $p < 0.01$) term of the model. Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model ($\chi^2 = 15.553$, $p < 0.05$). The interaction for power was observed in the quadratic ($t_{3132} = -3.305$, $p < 0.001$), and the fifth order ($t_{1184} = 2.177$, $p < 0.05$) terms of the model. Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model ($\chi^2 = 26.419$, $p < 0.001$).

22 – 28 kHz USVs

We did not observe any significant Treatment*Experiment Stage interaction, nor any main effect of treatment on the number or the acoustic characteristics of 22 – 28 kHz USVs emitted during the anticipatory period. Although it is important to note that the lack of any effects is not particularly surprising due to the short duration of the anticipatory period resulting in very few total 22 – 28 kHz calls per animal (Control: 20.37 ± 7.09; EtOH: 25.37 ± 16.86) over the course of the experiment.

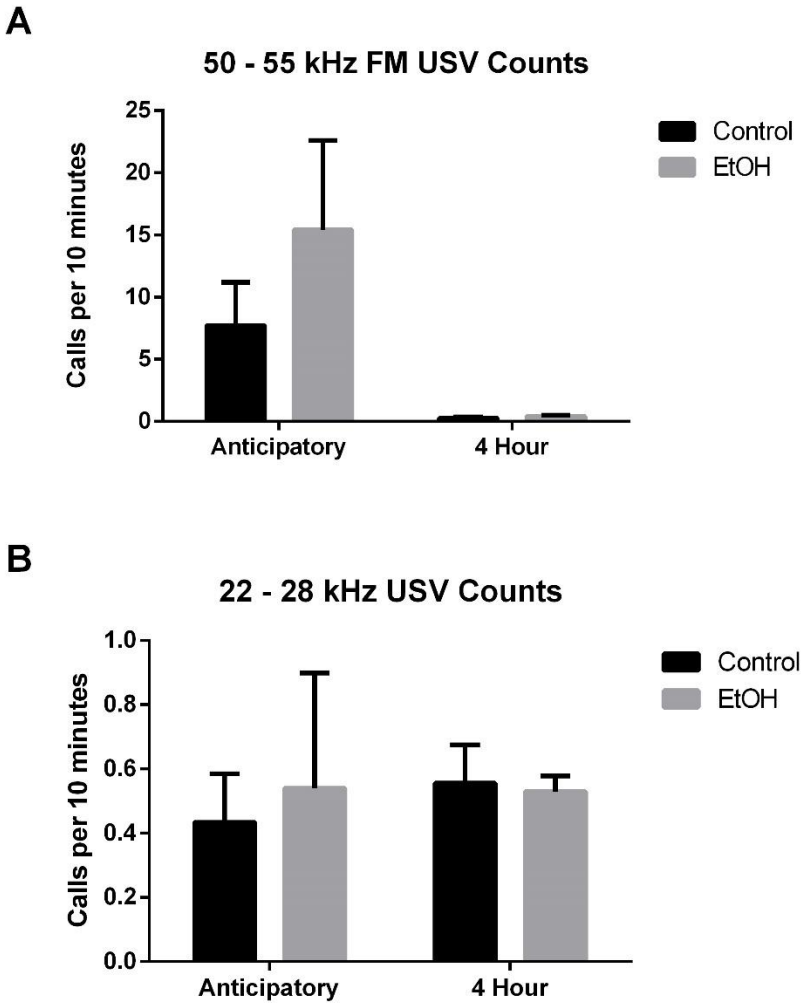
Similar to the anticipatory period results, we did not observe any significant Treatment*Experiment Stage interaction, nor any main effect of treatment on the number

of 22 – 28 kHz calls emitted during the 4-hour recording period (Figure 7A). However, there was a significant Treatment*Experiment Stage interaction for the mean frequency (Figure 7B), duration (Figure 7C), bandwidth (Figure 7D) and power (Figure 7E) of these calls. The significant interaction for mean frequency was observed in the linear (t_{30} = 2.314, $p < 0.05$), the fourth order (t_{16700} = -3.439, $p < 0.001$) and the sixth order (t_{16710} = 4.160, $p < 0.0001$) terms of the model. Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model (χ^2 = 76.167, $p < 0.0001$). The significant interaction for duration was observed in the quadratic (t_{1140} = -3.876, $p < 0.001$), cubic (t_{9871} = -9.498, $p < 0.0001$), fourth order (t_{16528} = -5.656, $p < 0.0001$) and the sixth order (t_{16118} = -3.632, $p < 0.001$) terms of the model. Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model (χ^2 = 128.44, $p < 0.0001$). The interaction for bandwidth was observed in the linear (t_{37} = 2.764, $p < 0.01$), cubic (t_{7163} = -2.080, $p < 0.05$) and the fourth (t_{1671} = -3.914, $p < 0.0001$) terms of the model. Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model (χ^2 = 27.261, $p < 0.001$). The interaction for power was observed in the cubic (t_{7721} = -4.043, $p < 0.0001$), fourth order (t_{16704} = -3.656, $p < 0.0001$), fifth order (t_{16375} = -4.256, $p < 0.0001$) and the sixth order (t_{16724} = -5.107, $p < 0.0001$) terms of the model. Removal of this interaction resulted in a significant reduction in the goodness-of-fit of the model (χ^2 = 51.133, $p < 0.0001$).

Illustration 2: Schematic of experimental procedures.

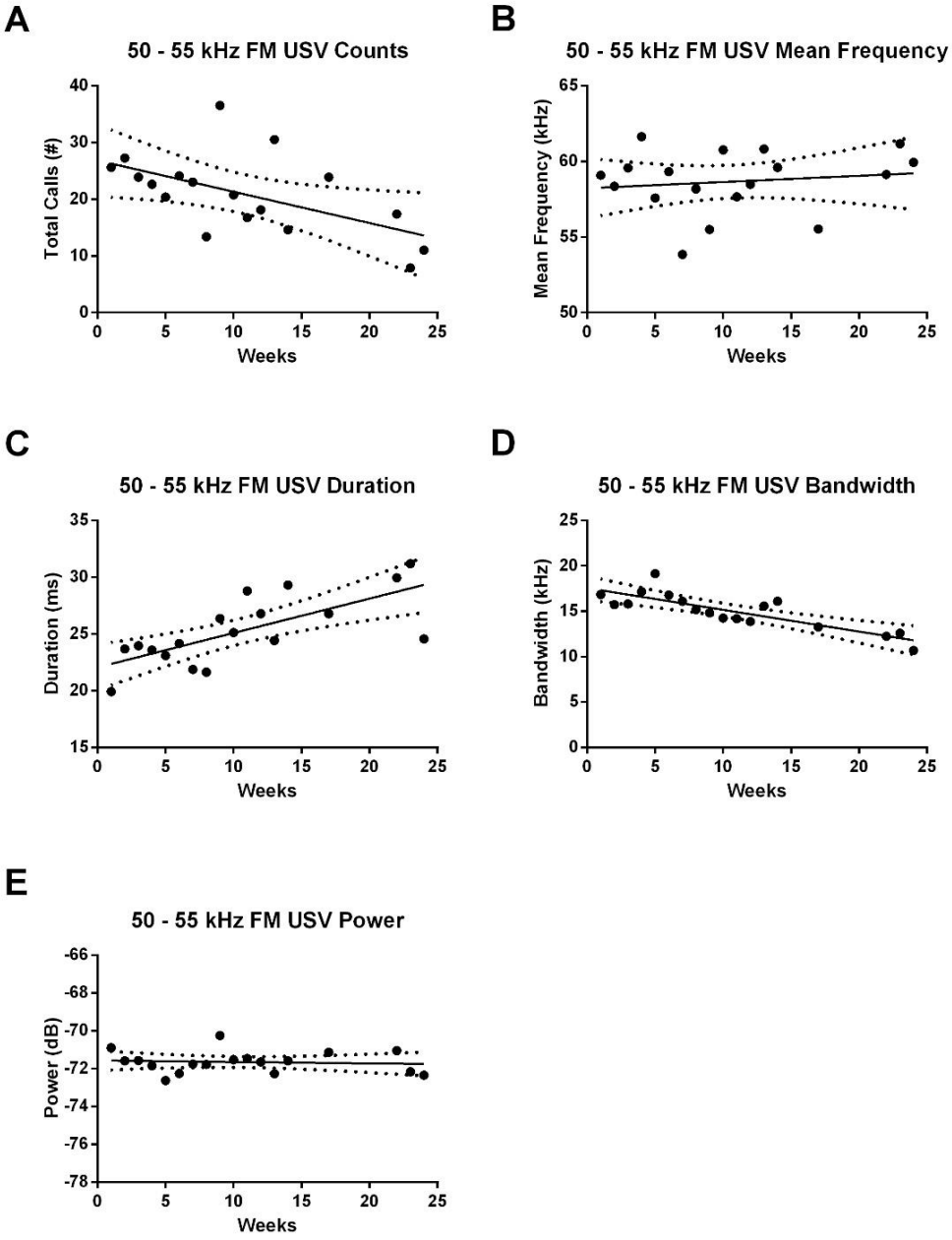
USVs were recorded from adult, male Long-Evans rats with variable access to 3-bottle choice EtOH (water, 15% EtOH, 30% EtOH) or water controls over a 24-week period.

Figure 16: Anticipatory vs. 4-hour counts.



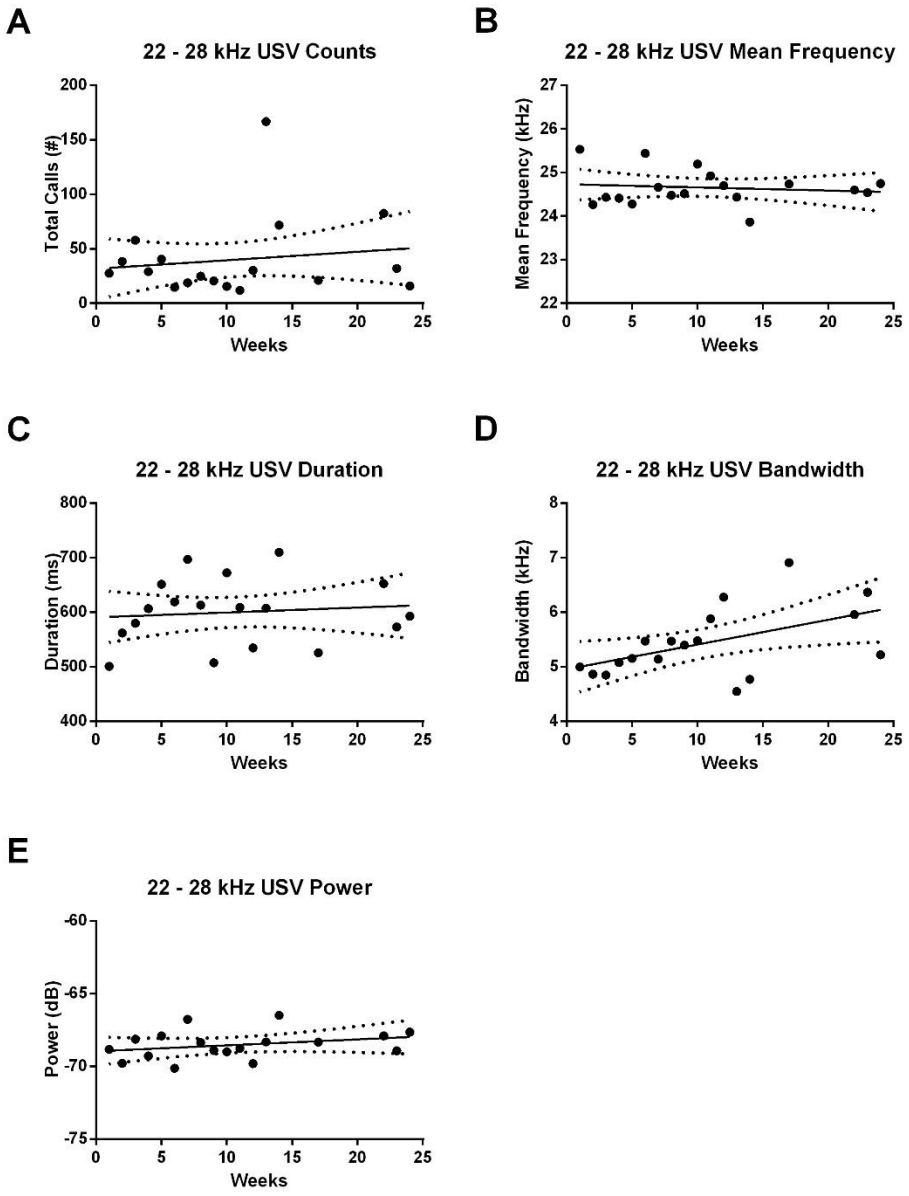
A) Rate of 50 – 55 kHz FM USVs emitted during the anticipatory period was significantly higher compared to the subsequent 4-hour session ($p < 0.05$). However, there was no difference in this rate between EtOH and control rats. B) There was no difference in the rate of 22 – 28 kHz USV emissions between the anticipatory or the 4-hour periods, nor between EtOH or water treated rats.

Figure 17: 50 – 55 kHz FM USV regression.



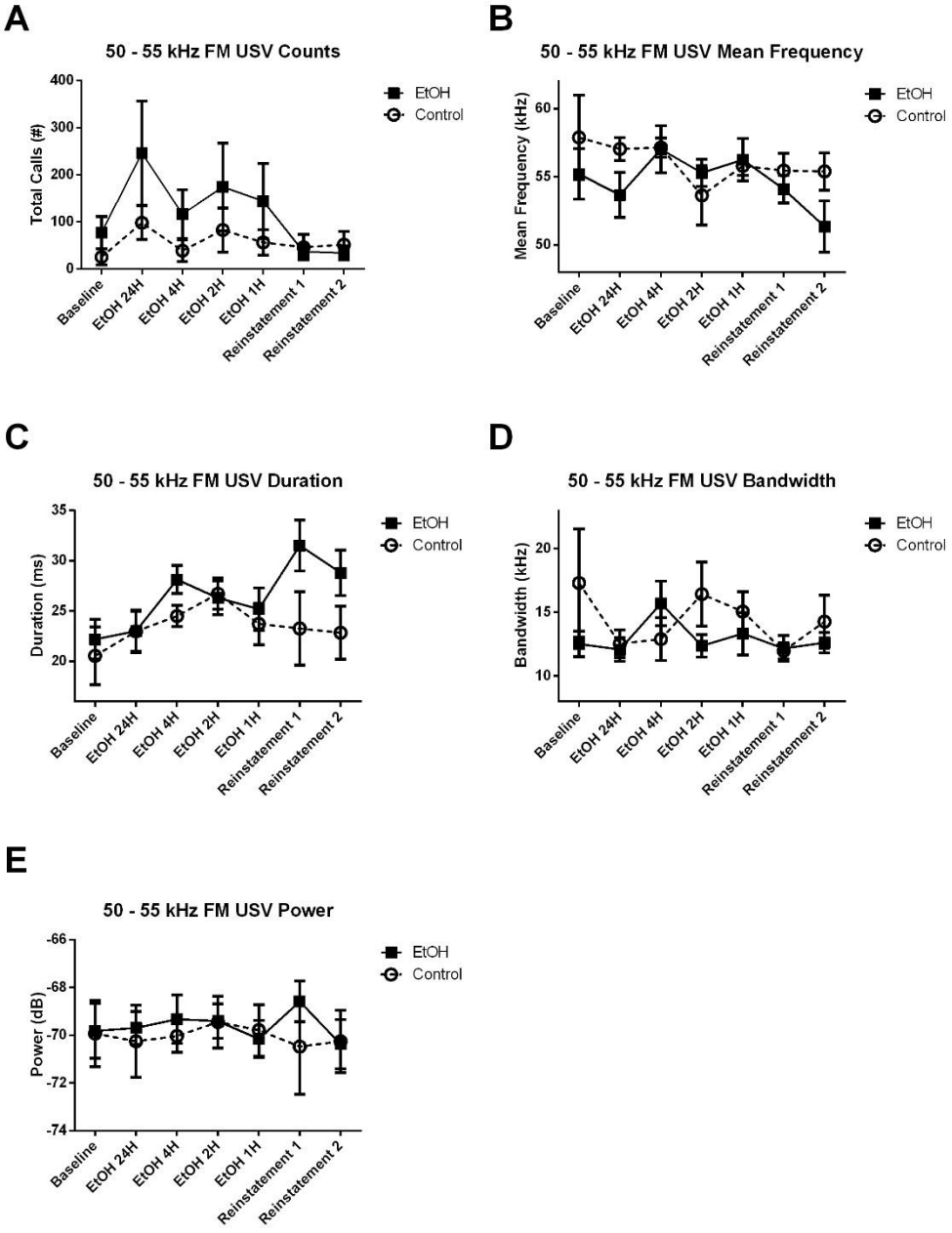
A) There was a significant reduction in total 50 – 55 kHz FM USV counts as the animals aged ($p < 0.05$). B) Mean frequency of 50 – 55 kHz FM USVs did not vary with age. There was an increase in C) duration, and decrease in D) bandwidth with age. E) Power did not vary with age.

Figure 18: 22 – 28 kHz USV regression.



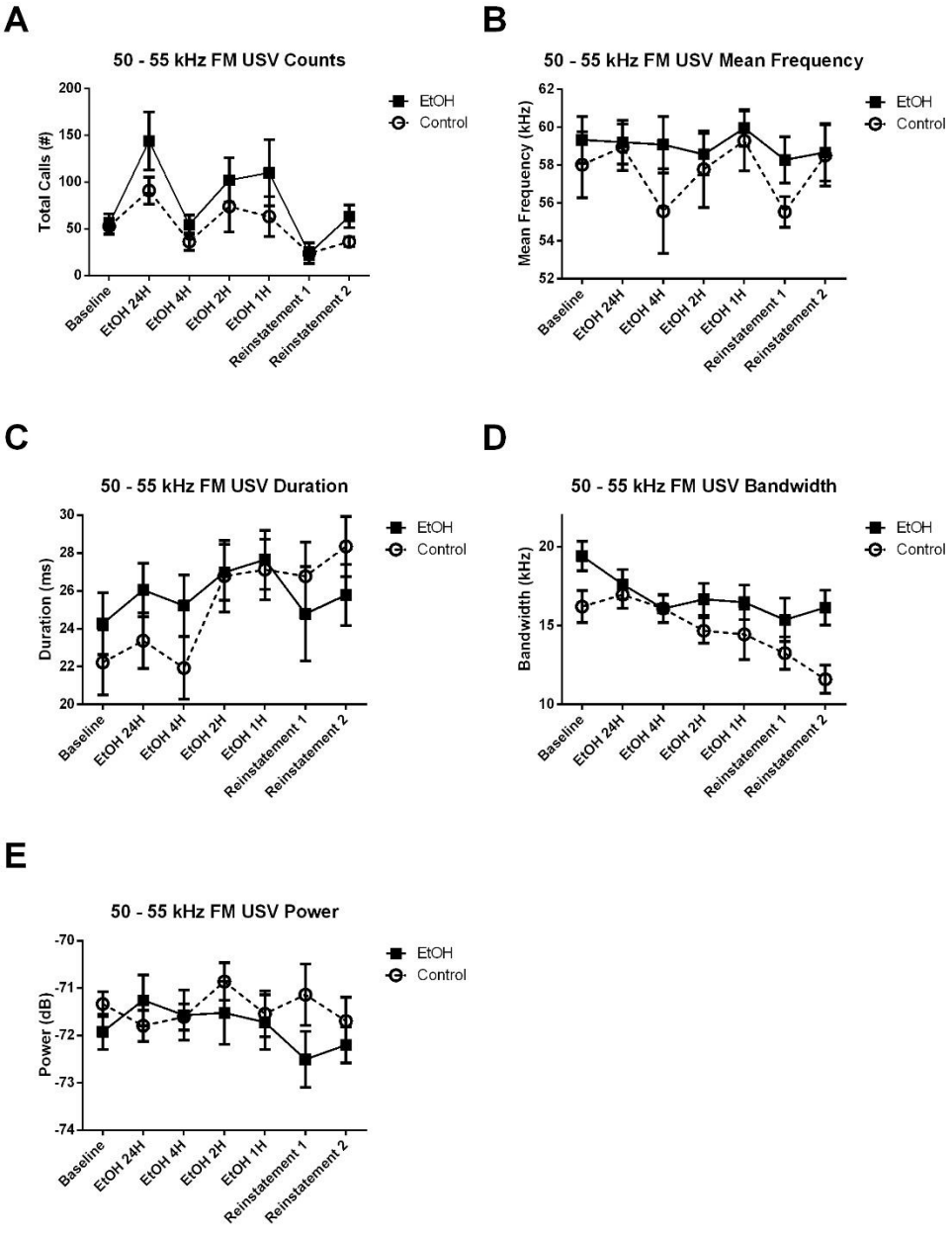
A) There was no change in total 22 – 28 kHz USV counts as the animals aged. B) Mean frequency or C) duration of 22 – 28 kHz USVs did not vary with age. D) There was an increase in bandwidth with age. E) Power of these calls did not vary with age.

Figure 19: Anticipatory 50 – 55 kHz FM USVs.



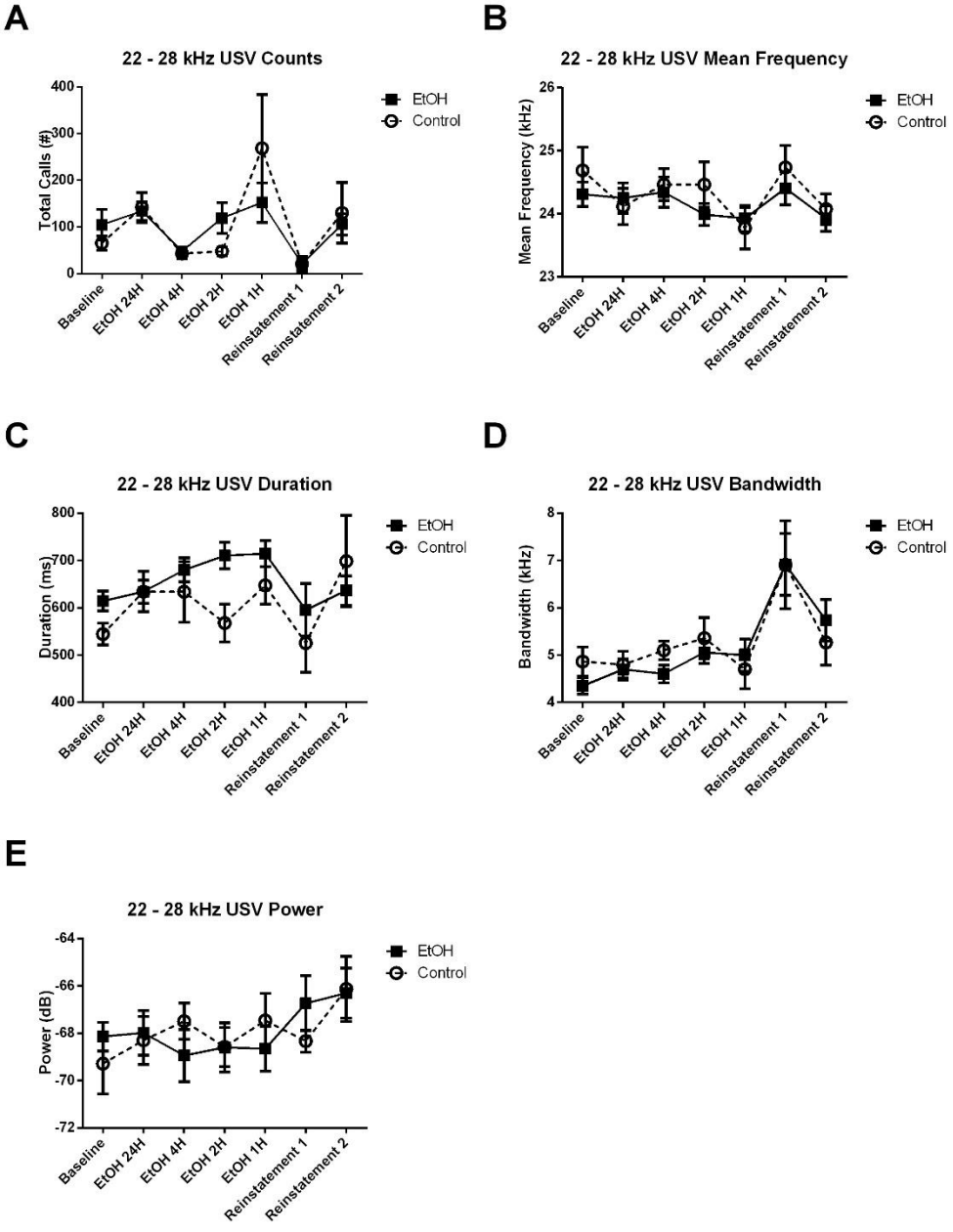
A) There was no Treatment*Experiment Stage interaction, nor any main effect of treatment on the number of 50 – 55 kHz FM calls. B) There was a significant Treatment*Experiment Stage interaction for mean frequency ($p < 0.0001$). There was no Treatment*Experiment Stage interaction, nor any main effect of treatment on the C) duration or D) bandwidth of these calls. E) There was a significant Treatment*Experiment Stage interaction for the power of these calls ($p < 0.001$).

Figure 20: 4-hour 50 – 55 kHz FM USVs.



A) There was no Treatment*Experiment Stage interaction, nor any main effect of treatment on the number of 50 – 55 kHz FM calls. B) There was a significant Treatment*Experiment Stage interaction for mean frequency ($p < 0.05$) of these calls. C) There was no Treatment*Experiment Stage interaction, nor any main effect of treatment on duration. There was a significant Treatment*Experiment Stage interaction for D) bandwidth ($p < 0.05$), and E) power ($p < 0.05$) of these calls.

Figure 21: 4-hour 22 – 28 kHz USVs.



A) There was no Treatment*Experiment Stage interaction, nor any main effect of treatment on the number of 22 – 28 kHz calls. B) There was a significant Treatment*Experiment Stage interaction for mean frequency ($p < 0.0001$), C) duration ($p < 0.0001$), D) bandwidth ($p < 0.001$), and E) power ($p < 0.0001$) of these calls.

DISCUSSION

This study examined the effect of age and ethanol exposure on spontaneous 50 – 55 kHz FM and 22 – 28 kHz USV emissions. Over the course of 24 weeks, we recorded USVs from adult male Long-Evans rats under alcohol-naïve, 24-hour EtOH access, 4-hour EtOH access, 2-hour EtOH access and 1-hour EtOH access conditions. The recording sessions began when the subjects were 2 months old and continued until they were approximately 8 months old. Therefore, we first assessed how USV counts and characteristics change over time and these rats grow in age and size. Next, we investigated the effects of ethanol exposure on USV transmission. From these experiments a number of interesting results emerged, which were consistent with our hypotheses that acoustic characteristics of USVs spontaneously emitted by male Long-Evans rats change as a function of age and that these changes are sensitive to ethanol exposure.

This study provided novel evidence that Long-Evans rats spontaneously emit 22 – 28 kHz and 50 – 55 kHz FM USVs. Here we also showed that Long-Evans rats emit increased amounts of 50 – 55 kHz FM, but not 22 – 28 kHz, USVs when initially places into the recording chamber before any alcohol, food or water is introduced. This result suggests that 50 – 55 kHz FM USVs may be associated with anticipatory and exploratory behavior in these rats. Moreover, although both control and ethanol treated animals exhibited increased calling during the 10-minute 'anticipatory' periods, only the ethanol group had a significantly higher emission rate during the anticipatory period than during the remaining 4-hour session. This high rate may be due, in part, to an anticipation of ethanol and is consistent with previous work showing similar increases in 50 – 55 kHz FM USVs in anticipation of cocaine or alcohol self-administration (Ma et al., 2010; Maier et al., 2010; Buck et al., 2014a).

Interestingly, although we did not see any age-dependent changes in these spontaneously emitted anticipatory calls, here we provide novel evidence of a decrease in the 50 – 55 kHz FM, but not 22 – 28 kHz, USV emissions as these rats mature. We also show that as these rats get older the spontaneously emitted 50 – 55 kHz calls increase in duration, but decrease in complexity as measured by the bandwidth of these calls. This decrease in bandwidth with age is in line with previously published literature showing a reduction in the complexity of 50 – 55 kHz calls emitted by 32-month old geriatric rats (Basken et al., 2012). Here we show that such a decline in call bandwidth begins as early as 6-months in male Long-Evans rats. On the other hand, the bandwidth of spontaneously emitted 22 – 28 kHz calls increased with age. It is interesting that these two USV subtypes, which represent opposing motivational and emotional states undergo opposing age-related changes. These results might suggest antagonistic changes in the neurotransmitter systems that underlie 22 – 28 kHz and 50 – 55 kHz FM calls. However, more direct experiments are needed to further explore this hypothesis.

Lastly, we used linear mixed models to examine the effect of ethanol treatment on the counts and acoustic characteristics of spontaneously emitted 50 – 55 kHz FM and 22 – 28 kHz USVs. Here we report that although ethanol exposure did not significantly alter the total number of spontaneous USVs emitted as compared to the control group, ethanol exposure differentially modulated the effect of age on the acoustic characteristics of both 50 – 55 kHz FM and 22 – 28 kHz USVs. We found significant ethanol treatment by time interactions on the mean frequency, bandwidth and power, but not duration, of 50 – 55 kHz FM USVs. We are also significant ethanol treatment by time interactions on all four of these acoustic characteristics of spontaneously emitted 22 – 28 kHz USVs. These results are not particularly surprising since acute and chronic ethanol exposure affects the activity of dopaminergic and cholinergic systems (Yoshimoto et al., 1992; Vengeliene et

al., 2008; Wang et al., 2009; Prasad et al., 2010; Jung et al., 2011; Rahman et al., 2015), which are known underlie the transmission of 50 – 55 kHz FM and 22 – 28 kHz USVs (Burgdorf et al., 2007; Litvin et al., 2007; Simola et al., 2012; Brudzynski, 2015; Wöhr et al., 2015). These results suggest that USVs, in serving as functional marker of dopaminergic and cholinergic activity, may provide useful information about the neurochemical changes produced by acute and chronic ethanol exposure. In summary, this study adds to the breadth of existing literature highlighting the utility of USVs as important non-invasive, behavioral correlates of the neurological changes induced by alcohol and other drugs of abuse.

Chapter 7: Conclusions

Excessive alcohol consumption has a vast negative impact on personal and societal health and well-being. Alcohol is by far the most commonly used drug of abuse and the range of inter-individual alcohol consumption levels is very broad. Of all individual who report consuming alcohol at least once within the past year, ~40% engage in binge drinking, while only ~13% report excessive or hazardous alcohol use. Although a relatively small proportion of alcohol consumers transition to hazardous use and dependence, the total number of such individuals is very high at approximately 16 million. The goal of the present dissertation work was to explore the various factors that drive inter-individual variability in alcohol consumption and alcohol abuse. It is well known that both genetic and environmental factors play an important role in the development of AUDs, with genetic factors accounting for nearly 50% of the observed variability. Specifically, it has been shown that genetic variation in the major neurotransmitter systems plays an important role in alcohol use and abuse. Here we identified behavioral phenotypes, which can serve as potential biomarkers for underlying variability in neurotransmission. Rodents also show a wide range of drinking levels, which are comparable to human subjects. In order to identify the neural correlates underlying the inter-individual differences in alcohol consumption levels in these rats, we investigated the use of basal ultrasonic vocalization emissions as functional markers of underlying neurotransmission and predictors of future alcohol-consumption.

SEX DIFFERENCES IN ALCOHOL USE AND USV PROFILES

In the first two studies, we explored sex-differences associated with alcohol consumption in the selectively bred high-alcohol drinking (HAD-1) rat line. Sex and

gender specific differences in alcohol consumption patterns are observed in humans and rodents alike. Although historically human females consume lower levels of alcohol than their male counterparts, the converse is true for rodent subjects (Almeida et al., 1998; Maldonado-Devincci et al., 2010; Nieto and Kosten, 2017). This discrepancy in relative alcohol consumption levels between human and rodent females may be due to a variety of external factors and social constraints. Indeed, recent data suggest that men and women are becoming more similar in alcohol abuse patterns (Substance Abuse and Mental Health Services Administration and Administration, 2014), such as alcohol drinking frequency and driving while intoxicated (White et al., 2015). Moreover, studies show that human females exhibit enhanced vulnerability for the negative consequences of alcohol consumption including higher intoxication and blackout rates (Trillo et al., 2012; Schuckit et al., 2016; Cheng and Anthony, 2017). In line with established literature, we observed that female HAD-1 rats consumed higher levels of alcohol than male HAD-1 rats. In the first study, we explored sex-specific USV profiles that might be associated with the observed differences in alcohol-consumption between male and female HAD-1 rats. We found that while both male and female HAD-1 rats emit large number of unprovoked 50 – 55 kHz FM and 22 – 28 kHz USVs, females emitted more 50 – 55 kHz FM USVs than males. Moreover, binomial logistic regression and linear mixed models revealed significant sex differences in the mean frequency, duration, bandwidth and power of 50 – 55 kHz FM and 22 – 28 kHz USVs that were differentially modulated by EtOH exposure. These results provide support for the use of USVs as reliable markers of sex differences in HAD-1 rats and demonstrate the sensitivity of USV acoustic parameter analyses for detecting alcohol and alcohol experience-induced alterations.

In the second study, we further extended this work by investigating differences in innate exploratory behaviors and working memory in male and female HAD-1 rats using

a one-trial novel object recognition task (ORT). Female HAD-1 rats showed increased exploratory activity, as measured by rearing behavior and object investigation time, during the initial training phase of ORT. Moreover, we observed a significant correlation between object investigation time, total rearing behavior and alcohol consumption in these rats. Together these results provide direct evidence that spontaneous ultrasonic vocalization emissions and novelty-seeking behavior on ORT can serve as predictors for the propensity to consume alcohol in male and female HAD-1 rats.

DIFFERENCES IN USV PROFILES OF HIGH- AND LOW-ALCOHOL-DRINKING RAT LINES

Since male and female rats differ significantly in their alcohol consumption patterns, it is possible that the observed differences in basal USV emissions may be associated with the propensity for alcohol consumption in the selectively-bred HAD-1 rats. To directly address this question, we compared the spontaneous USV profiles of male HAD-1 rats with their low-alcohol-drinking (LAD-1) counterparts in the third study. We found clear differences in the acoustic characteristics of 50 – 55 kHz FM and 22 – 28 kHz USV calls between alcohol-naïve HAD-1 and LAD-1 rats. Moreover, we applied machine-learning algorithms to demonstrate that a linear combination of these USV acoustic characteristics (i.e. mean frequency, duration, bandwidth and power) accurately discriminates between HAD-1 and LAD-1 rats exclusively on the basis of USV data collected in an alcohol-naïve state. In a separate but related experiment, our lab showed similar differences in basal USV acoustic profiles between selectively-bred alcohol-preferring (P) and alcohol non-preferring (NP) rats (Reno et al., 2017). Together these results provide direct and novel evidence that acoustic phenotypes of spontaneously emitted USVs can be used to discriminate between subjects predisposed towards high or low levels of alcohol consumption.

While the selectively bred P/NP and the HAD-1/LAD-1 rat lines are well-established and commonly used preclinical models of alcohol abuse, they do have some drawbacks. Due to the small populations typically used in the selective-breeding process, it is possible for inbreeding to occur. Inbreeding could result in the amplification of random genetic phenotypes that are unrelated to the selection criteria. Thus, the differences observed in the USV profiles of these rats, could simply be a by-product of random genetic drift resulting from the selective-breeding process. However, since USV differences are observed between both P/NP and HAD-1/LAD-1 rat lines that originate from distinct breeder strains it is unlikely that observed difference in the USV acoustic profiles of these rat lines were simply due to random genetic drift. Nonetheless, to assess the capacity of USVs to predict alcohol consumption behaviors in an outbred rat line, unencumbered by the potential limitations of the selective-breeding process, we conducted a meta-synthetic analysis where we directly compared data from the P/NP and the HAD-1/LAD-1 studies with new data collected from the outbred Long-Evans rats.

USV PROFILES PREDICT PREDISPOSITION FOR EXCESSIVE ALCOHOL USE

In this fourth study, we collected spontaneous USV emission data from alcohol-naïve, male Long-Evans rats and used machine-learning models to assess the similarity of the USV emitted by Long-Evans rats with the P/NP and the HAD-1/LAD-1 pairs. Using two separate models, we designated the linear combinatorial scores of the USV acoustic characteristics of the P rats and the NP rats (as well as the HAD-1 rats and the LAD-1 rats) as the high and low end of an alcohol consumption spectrum. The Long-Evans rats were then scored along this spectrum in terms the similarity of the USV scores with either P or the NP rats (or HAD-1 or LAD-1 rats). The scores generated using this method were then used to build a model that predicted the propensity for future alcohol

consumption in these rats. Following the USV recording experiments, the rats received 4 weeks of 24-hour EtOH access sessions (3 days/week) and their alcohol consumption during these sessions was compared to the model predicted alcohol consumption levels. We found that both the P-LE-NP and the HAD-LE-LAD models predicted alcohol consumption levels with a high degree of accuracy and that the resulting prediction was significantly correlated with future consumption levels. This study provided the first evidence for the utility of acoustic characteristics of USVs spontaneously emitted in an alcohol-naïve state in predicting alcohol consumption behaviors in an outbred strain of rats.

It is interesting to note that both models produced accurate predictions of alcohol consumption, despite the fact that the P/NP and the HAD-1/LAD-1 lines are known to have very distinctive behavioral and neurobiological profiles (Bell et al., 2017). These replicate lines show differential expression patterns for many of the neurochemical substrates that are thought to underlie the motivation for alcohol consumption. Yet they do share similarities in alcohol consumption levels as well as in their susceptibility for the rewarding and reinforcing properties of alcohol (McBride and Li, 1998; Murphy et al., 2002). Since there are known differences in the neurotransmitter systems associated with alcohol consumption and USV emissions (i.e. the dopaminergic and the cholinergic system) between P/NP and HAD-1/LAD-1 lines, it might appear paradoxical at first that data from both of these lines could be used to generate highly accurate predictive-models for alcohol consumption in the LE rats. However, it is important to note here that although there may be differences in the absolute expression patterns of neurotransmitter systems (and other neurobiological substrates) across the P/NP and HAD-1/LAD-1 rat lines, these models were generated to maximize the separation achieved using the relative

differences in the expression of neural substrates between the high- and low-drinking subject, within each replicate line.

These results do not suggest correspondence in the neural and genetic make-up of the HAD-1 vs. P or the LAD-1 vs. NP rat lines. Indeed, an examination of the acoustic characteristics actually shows that while the P rats emit 22 – 28 kHz USVs with a higher mean frequency, longer duration and a wider bandwidth than the NP rats, the HAD-1 rats made calls with a shorter duration than the LAD-1 rats and no further differences were seen in the mean frequency or bandwidth of these calls. Moreover, while we did not observe any differences in the acoustic characteristics of 50 – 55 kHz FM USVs between the P and the NP rats, the HAD-1 rats made such calls with a high mean frequency, longer duration and narrower bandwidth than the LAD-1 rats. These results are in line with the hypothesis that distinct neural substrates drive alcohol consumption behaviors in the P/NP and HAD-1/LAD-1 rat lines. It is for this reason that we built two separate models independently comparing the Long-Evans rats with the high- or low-drinking subjects in each of these replicate rat lines. Thus, these models can take advantage of the relative differences in the neural and genetic make-up of these selectively bred rats and rank Long-Evans rats in terms of their similarity to either the high- or the low-drinking subjects. Since the models are designed to maximize alcohol-associated differences within each replicate pair, they are not confounded by known differences in the underlying neural substrates across these lines. One interesting implication of these results is that while differential biological factors may drive alcohol consumption behaviors in the P and the HAD-1 rats, a combination of factors represented in both of these lines may be responsible for driving alcohol consumption in the Long-Evans rats. This hypothesis is intuitive because although the different selective-breeding processes behind the generation of the P/NP and HAD-1/LAD-1 rat lines may have resulted in the

amplification of specific and distinct neurobiological substrates associated with alcohol consumption, it is likely that the outbred progenitor strains expressed a wider array of such substrates that underlie alcohol consumption. Future studies will need to directly compare neurotransmission across these five rat lines in order to test this hypothesis.

AGE AND ETOH MODULATE USV ACOUSTIC CHARACTERISTICS

In the fifth study included in this dissertation, we conducted a 24-week longitudinal assessment of the effects of age and ethanol exposure on spontaneous USV transmission. We found that Long-Evans rats show age-dependent changes in the acoustic characteristics of spontaneously emitted 50 – 55 kHz FM and 22 – 28 kHz USVs, which were sensitive to modulation by ethanol exposure. Together these five studies highlight a new role for USVs as predictive biomarkers of alcohol-associated behaviors. Since USV transmission is sensitive to pharmacological manipulations of underlying dopaminergic and cholinergic activity, it is possible that basal USV emissions can serve as functional reflections of activity in these neurotransmitter systems. While, USVs are certainly not the only non-invasive biomarkers of consummatory behaviors, as evidenced by the ORT data, they are a vastly underutilized tool which can provide important information about the emotional and motivational status of rodent subjects.

DEVELOPING A NON-INVASIVE SCREEN FOR HIGH ALCOHOL CONSUMING LONG-EVANS RATS

Findings from our integrative study of USV profiles from five separate rats lines provided novel evidence that USV data collected from alcohol-naïve rats can predict future alcohol consumption in these rats. However, one limitation of this work is that this study employed a retrospective approach, such that the models generated to predict future alcohol consumption relied on empirically collected alcohol consumption values from the

same subjects. Therefore, in order to determine whether the model predictions are similarly accurate on subjects whose data are not already included, we could simply build machine-learning models using only the data from the HAD-1/LAD-1 or P/NP rat lines and determine whether these models could still be used to generate USV scores for alcohol-naïve Long-Evans rats and predict their future alcohol-consumption. This approach overcomes the tautological issue of building a model using data from the very subjects we seek to predict.

To test this idea, we built two separate models: one with the USV data from alcohol-naïve HAD-1/LAD-1 rats and another with the USV data from alcohol-naïve P/NP rats. We then used these models to calculate USV scores for alcohol-naïve Long-Evans rats for both the 50 – 55 kHz FM and 22 – 28 kHz USV categories. Using multiple-linear regression analyses we found that the USV scores generated using both models significantly correlated with, and were predictive of future alcohol consumption in Long-Evans rats (P/NP model: multiple $R = 0.7186$, $p < 0.01$; HAD-1/LAD-1 model: multiple $R = 0.7339$, $p < 0.01$). These results provide the first direct evidence that the models described here can serve as independent and non-invasive, *a priori* screens for the propensity of excessive alcohol consumption in Long-Evans rats. Although these models will certainly benefit from the inclusion of additional data points to further strengthen their predictive accuracy, the present work describes a screening method that can predict alcohol consumption in alcohol-naïve Long-Evans rats with reasonable accuracy. This screening method is a substantial development and promises to have far-reaching consequences for preclinical alcohol research.

USV ACOUSTIC CHARACTERISTICS AS BIOMARKERS OF NEURAL TRANSMISSION

Historically, the study of USVs has been hampered by the manually intensive and time-consuming analysis process, which requires nearly 4 hours of assessment for every 10 minutes of USV recordings. The development of automated USV analysis tools such as WAAVES (Reno et al., 2013; Reno and Duvauchelle, 2014), and other template-matching techniques (Barker et al., 2014), will allow for greater flexibility in terms of incorporating a USV component to the existing battery of rodent behavioral assays. Such tools will also help us move beyond simple and crude metrics such as total call counts, by allowing us to extract a set of high-dimensional acoustic characteristic data which provide us detailed information about the nature of each individual USV call. While call counts have been shown to be a reliable marker of stimulus-induced USV emissions, which are sensitive to experimental manipulations, total call counts for spontaneously emitted calls are far less reliable. In many of the studies presented here, we saw very few differences in the total number of spontaneously emitted 50 – 55 kHz FM and 22 – 28 kHz USV calls, yet substantial differences were observed in these acoustic characteristics of these calls.

It must be noted that we did not directly examine neurotransmission in relation to the differences observed in USV acoustic characteristics across different experimental groups in this set of studies. Therefore, this does beg the question whether USV acoustic parameters are also a reliable indication of underlying neurotransmission in the same way as total call counts, which are sensitive to direct pharmacological manipulations of the dopaminergic and cholinergic systems. Although future studies will need to directly investigate the relationship between neural transmission and USV acoustic characteristics to definitively answer this question, there is some evidence to suggest that changes in USV counts are generally accompanied by concomitant changes in the acoustic profile of

these calls. The two broad categories of USV calls described here (i.e. 50 – 55 kHz FM and 22 – 28 kHz USVs) can be further subdivided into a number of categories, such as short or long 22 – 28 kHz calls, or trill, ramp, step, or composite 50 – 55 kHz FM calls. Pharmacological manipulations of the underlying neurotransmitter systems also alter the composition of the call subtypes that are emitted in addition to the total call counts.

Indeed, unpublished data from a collaborative project in our lab shows that optogenetic activation of the ventral tegmental area dopaminergic neurons not only results in a massive increase in the total number of emitted 50 – 55 kHz FM USV calls, but this increase is also accompanied with enhanced emissions of more complex and complicated call subtypes, than the ones seen in the control or in naïve rats. Similar changes in USV acoustic characteristics in response to experimental manipulations of dopaminergic activity have also been reported by other groups (Wintink and Brudzynski, 2001; Brudzynski et al., 2011; Simola, 2015). Moreover, the cholinomimetic drug carbachol has been shown to alter the acoustic characteristics of 22 – 28 kHz calls as well (Brudzynski, 1994). These results, coupled with our findings that USV acoustic characteristic data can be used to distinguish between, male and female HAD-1 rats, as well as, high- and low-drinking rat lines, despite no clear and present differences in total call counts speaks to the sensitivity of these metrics.

FUTURE DIRECTIONS

Although the work described in this dissertation only focuses on a preclinical model of alcohol abuse, the framework outlined here can be easily generalized to a wide variety of other neurological disorders. Studies utilizing USV data range from examination of neurodegeneration in models of Parkinson's disease (Johnson et al., 2015), aphasia in models of stroke (Pan et al., 2014), as well as, affective status in models

of depression (Kroes et al., 2007) and pain (Oliveira and Barros, 2006). Moreover, the approach of establishing a spectrum using data collected from rat lines selectively-bred could very easily be applied to other emotional and motivational phenotypes, for which such lines exist. Some examples include rat lines selectively bred for high- or low-levels of anxiety, impulsivity, or aggression (Steimer and Driscoll, 2003; Veenema and Neumann, 2007). Similar to the methodology described here, future studies could use USV data collected from these selectively bred rat lines to establish a spectrum upon which USVs from outbred rat strains such as Wistar, Long-Evans or Sprague-Dawley rats can be assessed to make predictions about the underlying psychological phenotypes.

In addition, using USV profiles of selectively bred high- and low-alcohol consuming rats, we have constructed screening methods that can accurately predict alcohol consumption in alcohol-naïve Long-Evans rats. In addition to its significance in the field of preclinical alcohol research, these findings may generalize to other drugs of abuse. If future studies confirmed the utility of these models for additional drugs of abuse, this screening method would have high impact for the drug addiction field.

In summary, the work presented in this dissertation highlights the versatility of high-dimensional USV data as sensitive and reliable behavioral biomarkers. The adoption of automated USV analysis tools, in combination with advanced statistical techniques designed to maximize the information that can be ascertained from such high-dimensional datasets can unlock the true potential of this relatively simple, yet powerful, behavioral tool. The work presented here lays a solid foundation for future work in this fields and provides a framework which can be emulated by future studies which aim to follow this exciting field of work.

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Vita

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