Sex-specific ultrasonic vocalization patterns and alcohol consumption in high alcohol-drinking (HAD-1) rats

Mittal, N[a,b], Thakore, N[a,b], Bell RL[e], Maddox, WT[c], Schallert T[b,d], Duvauchelle, CL[a,b]

[a] The University of Texas at Austin, College of Pharmacy, Division of Pharmacology and Toxicology, 2409 University Avenue, Stop A1915, Austin, TX 78712, USA

[b] Waggoner Center for Alcohol and Addiction Research, The University of Texas at Austin, 2500 Speedway, Stop A4800, Austin, TX 78712, USA

[c] Cognitive Design and Statistical Consulting, LLC, Austin, TX 78746, USA

[d] The University of Texas at Austin, College of Liberal Arts, Behavioral Neuroscience, 108 E. Dean Keeton, Stop A8000, Austin, TX 78712, USA

[e] Institute of Psychiatric Research, Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN 46202, USA

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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 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 [1], such as alcohol drinking frequency and driving while intoxicated [2]. 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 [3], and are more susceptible to the negative consequences of alcohol, such as cirrhosis, alcoholic liver injury and alcoholic cardiomyopathy [4–6].

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 [7] and enhanced first-pass metabolism of alcohol and its metabolites [8]. Moreover, research indicates that alcohol may modulate hormonal levels and function through its effects on both the hypothalamus and the gonads [9]. 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 [10–12]. 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 [13,14]. 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 [15,16]. 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 [16–18]. USVs have received increased attention in drug abuse studies because administration of cocaine [19], amphetamine [20] and drug-associated cues [21,22] 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 [23] 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 [24]. 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 [25–27].

Previous studies examining acoustic characteristics have done so by averaging across all of the USVs of a given type emitted by each animal [26,28–31]. 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 [32]. 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 [26] 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 [33,34] 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 (Figure 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" [32]. 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 (BLR). While LMM focuses on each acoustic property in isolation, we applied binomial logistic regression 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 [27]. 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[1] 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.

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.

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[1] 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.

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 ($p < 0.05$, $t_{587.90} = 2.330$; Figure 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 (Figure 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 Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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, χ^2 =9.7696) and a main effect of sex for the Control group (p < 0.05, χ^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 (Figure 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 (Figure 4d). Removal of this effect resulted in a significant reduction in the goodness-of-fit of the model (p < 0.01, χ^2 = 15.271). Independent assessment of the two treatment groups revealed a significant Sex*Time interaction for both EtOH (p < 0.0001, χ^2 =73.445) and Control (p < 0.0001, χ^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 (Figure 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 (Figure 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 (Figure 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 ($p < 0.0001$, χ^2 =120.79) and Control ($p < 0.0001$, χ^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 (Figure 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 ($p < 0.0001$, χ^2 =200.73) and Controls ($p < 0.0001$, χ^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 ($F_{1,15} = 12.52$, $p < 0.01$; Figure 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 ($F_{1,15} = 13.93$, $p < 0.01$; Figure 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 (Figure 6c). Though there was a significant increase in total fluid consumption over time for all groups ($F_{11,275} = 18.48$, $p < 0.0001$).

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 [26], 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 [15,17,35–37]. Since USVs are known to reflect real-time activity in dopaminergic and cholinergic neurotransmitter systems [38–46], USV counts have served as useful assessments of emotional responses during drug use and withdrawal [19,20,24]. 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 [47,48],

and template matching approaches [49,50] 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 [25,27]. 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 [10,12,51,52]. 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 [26]. 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) [53] 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) [25,27]. 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.

Table 1

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.

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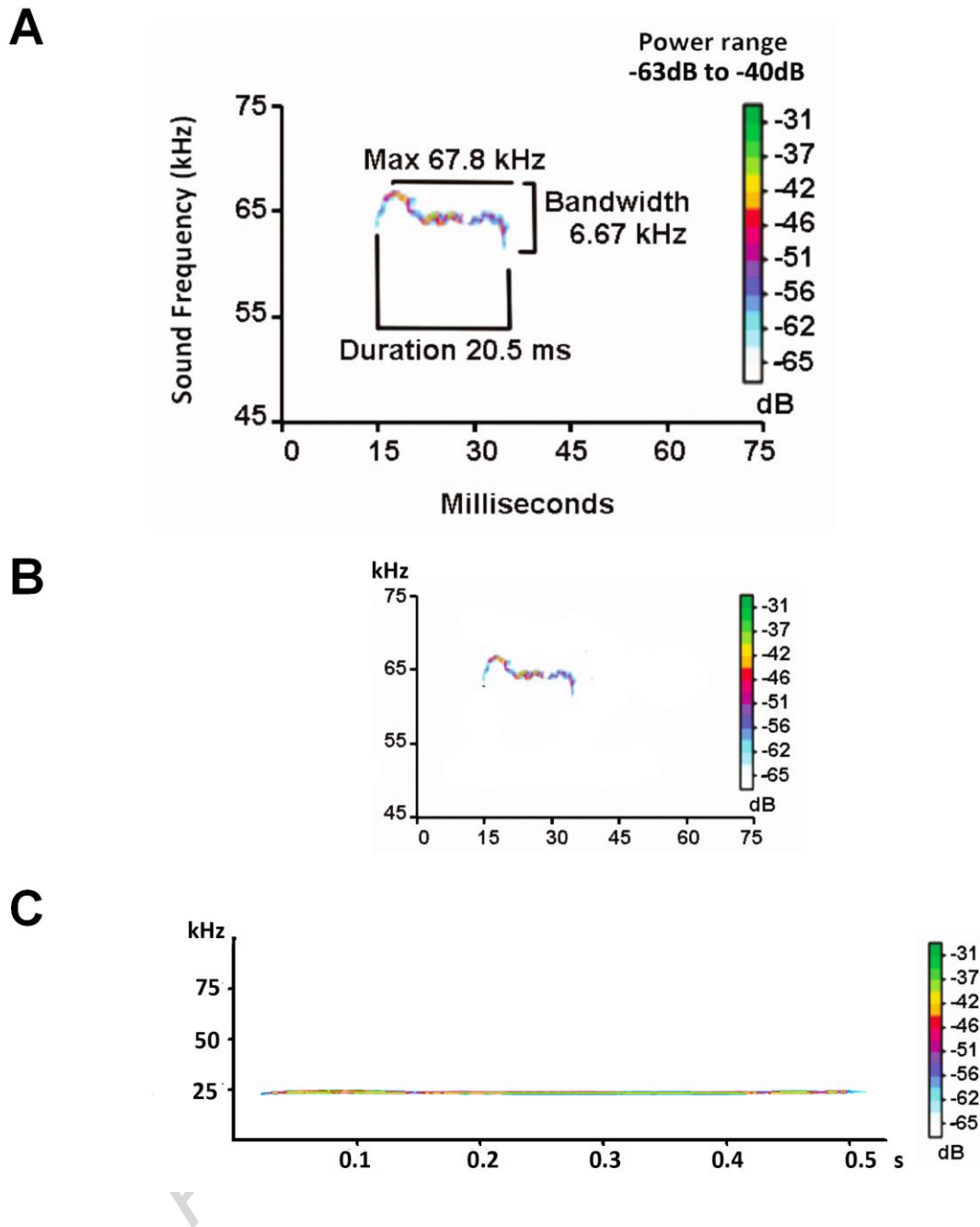
Figure 1: Schematic of representative spectra for different USV subtypes.

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

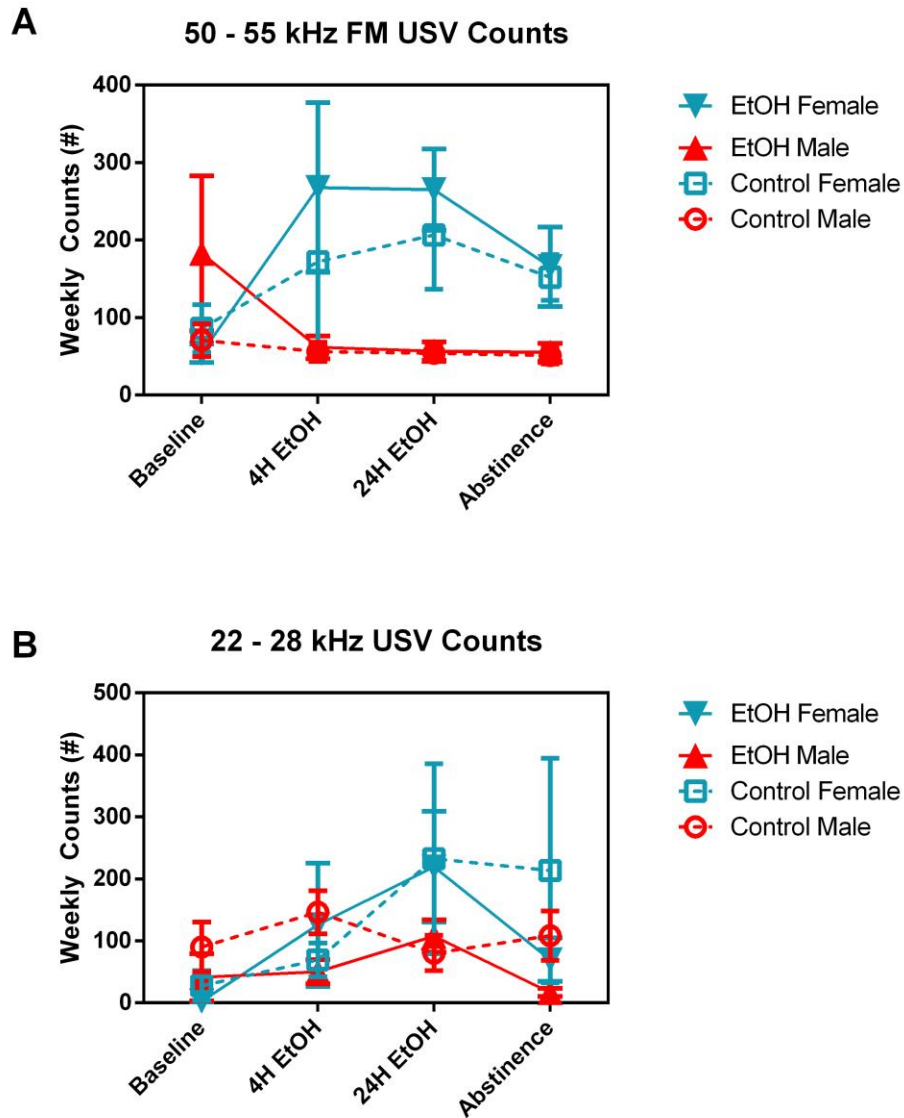modulated (FM) 50 – 55 kHz USV **C)** Example of a 22 – 28 kHz USV

A

50 - 55 kHz FM USV Counts

B

22 - 28 kHz USV Counts

Figure 2: 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.

A

50 - 55 kHz FM USV Classification Accuracy

B

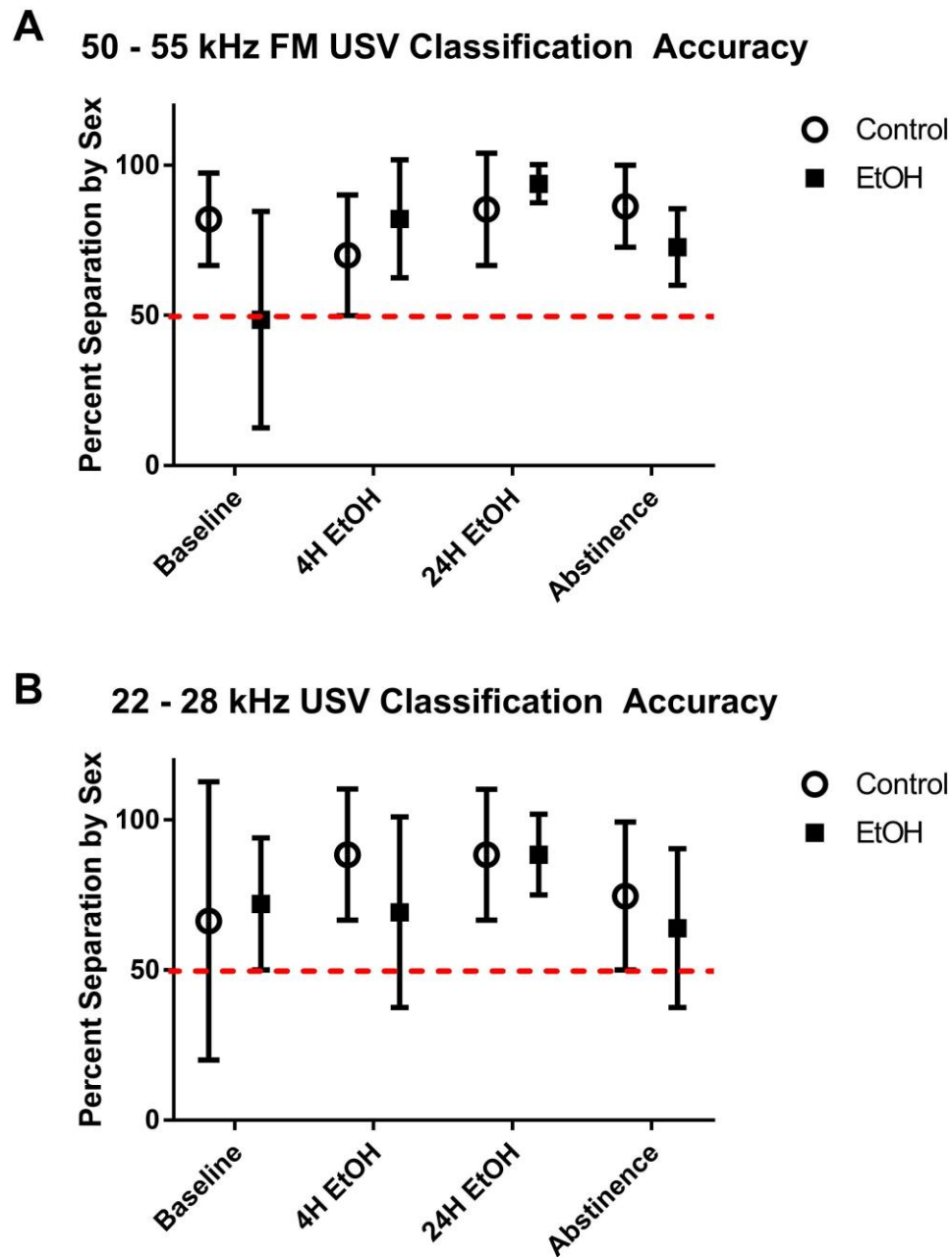### 22 - 28 kHz USV Classification Accuracy

Figure 3: Binomial logistic regression analysis: 50 – 55 kHz FM and 22 – 28 kHz USV acoustic characteristics

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.
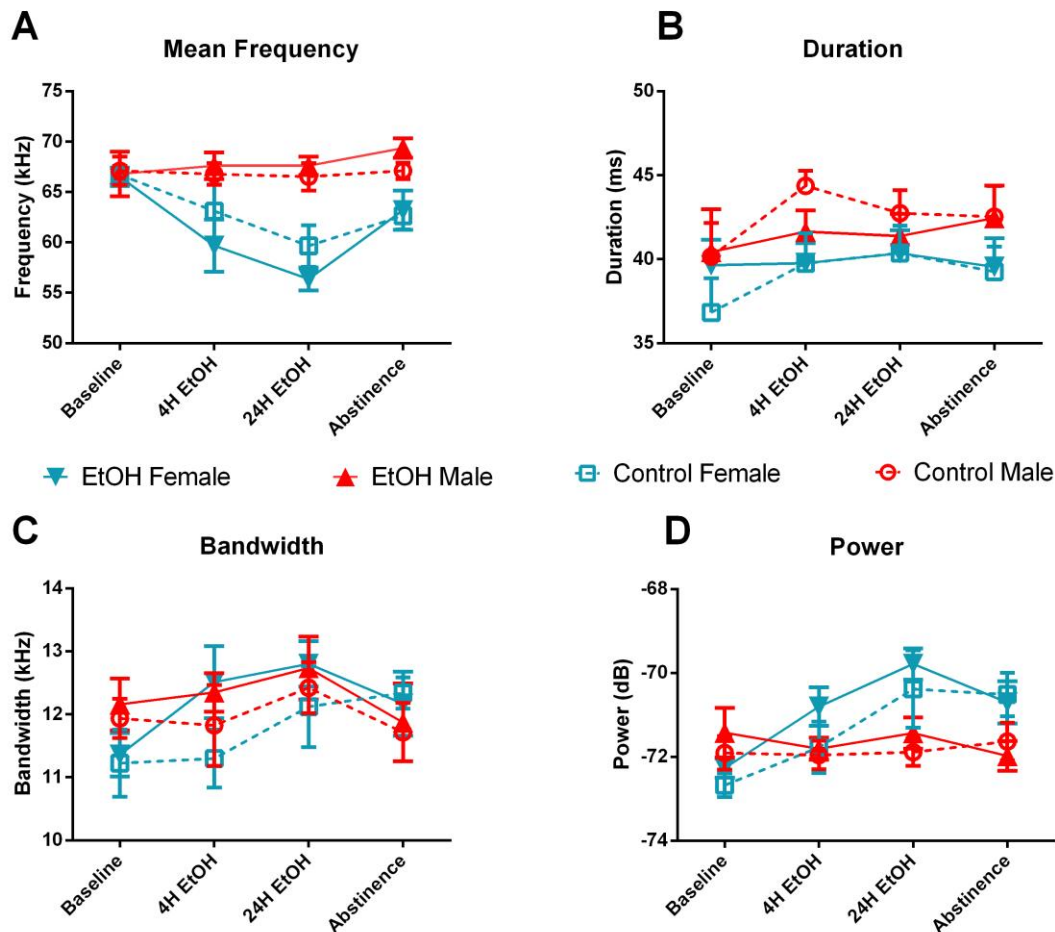
Figure 4: 50 – 55 kHz FM USV acoustic characteristics of male and female HAD-1 rats.

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 $\pm$ sem for each USV acoustic characteristic are reported.
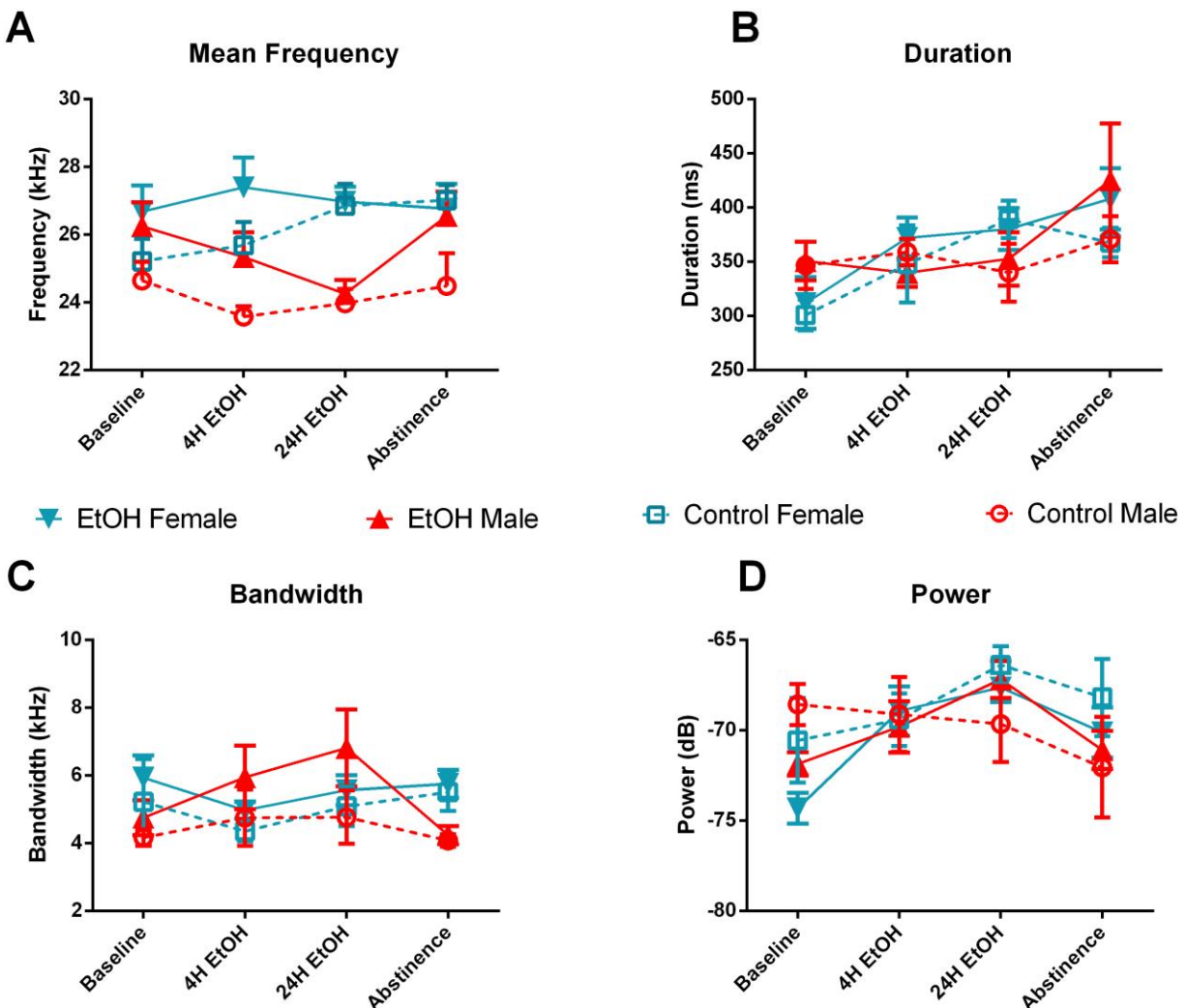
Figure 5: 22 – 28 kHz USV acoustic characteristics of male and female HAD-1 rats.

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 $\pm$ sem for each USV acoustic characteristic are reported.

A 4-Hour EtOH Consumption

B 24-Hour EtOH Consumption
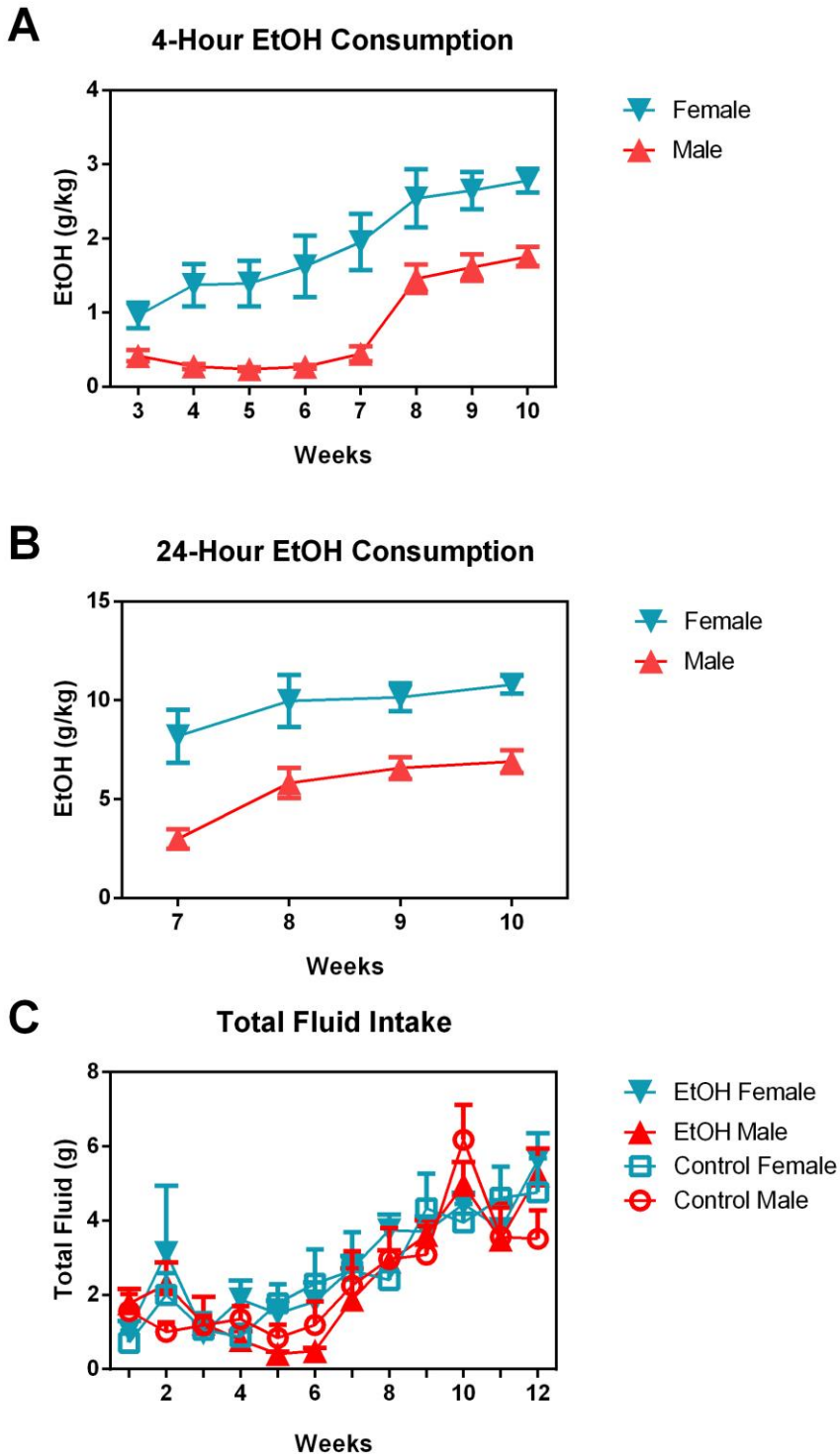
C Total Fluid Intake

Figure 6: 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.

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