Published in final edited form as:

Alcohol Clin Exp Res. 2017 April ; 41(4): 786–797. doi:10.1111/acer.13344.

Negative affect-associated USV acoustic characteristics predict future excessive alcohol drinking and alcohol avoidance in male P and NP rats

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Abstract

Background—Negative emotional status and adverse emotional events increase vulnerability to alcohol abuse. Ultrasonic vocalizations (USVs) emitted by rats are a well-established model of emotional status that can reflect positive or negative affective responses in real-time. Most USV studies assess counts, yet each USV is a multidimensional data point characterized by several acoustic characteristics that may provide insight into the neurocircuitry underlying emotional response.

Methods—USVs emitted from selectively bred alcohol-naïve and alcohol-experienced alcohol-preferring and non-preferring rats (P and NP rats) were recorded during 4-hr sessions on alternating days over 4 weeks. Linear mixed modeling (LMM) and linear discriminant analysis (LDA) were applied to USV acoustic characteristics (e.g., frequency, duration, power and bandwidth) of negative (22–28 kHz) and positive (50–55 kHz) affect–related USVs.

Results—100% separation between alcohol-naïve P and NP rats was achieved through a linear combination (produced by LDA) of USV acoustic characteristics of 22–28 kHz USVs, whereas poor separation (36.5%) was observed for 50–55 kHz USVs. 22–28 kHz LDA separation was high (87%) between alcohol-experienced P and NP rats, but was poor for 50–55 kHz USVs (57.3%). USV mean frequency and duration were the highest weighted characteristics in both the naïve and experienced 22–28 kHz LDA representations suggesting that alcohol experience does not alter the representations. LMM analyses of 22–28 kHz USV acoustic characteristics matched the LDA

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results. Poor LDA separation was observed between alcohol-naïve and experienced P rats for both 22–28 and 50–55 KHz USVs.

Conclusions—Advanced statistical analysis of negative affect-associated USV data predicts future behaviors of excessive alcohol drinking and alcohol avoidance in selectively bred rats. USV characteristics across rat lines reveal affect-related motivation to consume alcohol and may predict neural pathways mediating emotional response. Further characterization of these differences could delineate particular neurocircuitry and methods to ameliorate dysregulated emotional states often observed in human alcohol abusers.

Keywords

Linear Mixed Model; Linear Discriminant Analysis; Multidimensional Data; Selectively-bred rats; Alcohol-preferring and Non-preferring rats

INTRODUCTION

Heightened negative emotions, brought on by depression (Nurnberger et al., 2004), trauma (Weiss et al., 2015) and adversity (Sorocco et al., 2015, Chavez et al., 2015) result in a heightened risk of developing alcohol use disorders (AUDs). Ultrasonic vocalizations (USVs) are emitted by rodents in response to internal and external stimuli and are accepted as a reflection of the animal's affective state (Brudzynski, 2009, Brudzynski, 2007, Burgdorf et al., 2008, Duvauchelle et al., in press). USVs occur in two broad categories (and frequencies) representing positive (50–55 kHz) and negative (22–28 kHz) affect (Brudzynski, 2009, Brudzynski, 2007, Burgdorf et al., 2008) and are initiated through the mesolimbic dopamine (positive affect-related) (Brudzynski, 2007, Wright et al., 2012) and ascending cholinergic (negative affect-related) (Bihari et al., 2003, Brudzynski, 2014, Brudzynski et al., 2011) pathways.

Thus, changes in USVs may indicate alterations in the corresponding neural pathway. Currently, the vast majority of USV studies have focused primarily on the number of USVs elicited during experimental manipulations. However, each individual USV emission can be characterized by its values along a number of other acoustic dimensions such as frequency, duration, bandwidth and power. Alterations in these acoustic characteristics may provide a deeper understanding of neural pathways underlying emotional responses. For example, Brudzynski (1994) conducted a dose response study with the muscarinic agonist, carbachol, used to induce 22–28 kHz USVs, and found that the acoustic characteristics (e.g. duration, bandwidth, power) of 22–28 kHz USVs were sensitive to changes in dose. Other studies have begun to examine these acoustic characteristics (e.g. frequency, duration, bandwidth, power) more broadly (Inagaki et al., 2012, Brudzynski et al., 1991, Reno et al., 2015, Thakore et al., 2016). Thus, the information conveyed by USVs extends well beyond USV counts.

Preclinical research into alcohol use disorders has led to the development of divergent rat lines modeling high and low vulnerability for excessive alcohol consumption. The alcohol-preferring (P) and non-preferring (NP) rats are one such pair of bi-directionally selected rat lines selectively bred according to voluntary alcohol consumption (Bell et al., 2006, Bell et

al., 2012). The alcohol-preferring rat meets all of the criteria suggested for an animal model of alcoholism and displays multiple similarities with human alcoholics (McBride et al., 2014, Bell et al., 2012, Bell et al., 2016). In a recent study of USVs from alcohol-preferring P rats (Reno et al., 2015), we recorded USVs from naïve and alcohol-exposed P rats over the course of a daily 7-hour drinking-in-the-dark (DID) experiment (c.f., Bell et al., 2011). We discovered that both the naïve and the ethanol-experienced groups emitted unprovoked negative affect-related USVs, with some naïve P rats emitting over 200 in a seven-hour time period and some alcohol-exposed P rats emitting over 1000 in a seven-hour time span. We also found that control and ethanol-exposed P rats differed in the mean frequency of negative affect-related USVs, but not positive affect-related USV counts or acoustic properties (Reno et al., 2015).

The current report extends Reno et al. (2015) in a number of important ways. First, we include P and NP rat lines with rats from each line remaining ethanol-naïve or being treated with ethanol. A critical comparison here will be between P and NP alcohol-naïve controls to determine whether negative affect-related USVs are indicative of vulnerability to consume alcohol. Second, unlike Reno et al. (2015) we temporally separated the alcohol exposure from the USV recording session to better determine whether previous alcohol experience had short-or long-term effects on USV emotional phenotypes. Third, we used advanced statistics to explore the intricacies of the USV acoustic characteristics. Prior studies of USV acoustic characteristics averaged across all USVs characteristics of a given type for each animal then applied traditional ANOVA (Inagaki et al., 2012, Brudzynski et al., 1991, Thakore et al., 2016, Brudzynski, 1994, Reno et al., 2015). Reducing the data to a single number of central tendency (e.g. mean) loses much of the data's intricacies. To address this issue, we replaced ANOVA with linear mixed modeling (LMM) that models each USV and allows for unequal sample sizes and missing data points. Because it is likely that acoustic characteristics vary in a dependent manner (Brudzynski, 1994), we utilized linear discriminant analysis (LDA) to explore the collection of USV characteristics as a whole. A meaningful outcome of the current experiment would be the development of methods that can utilize emitted USVs to distinguish between rat populations that appear to be identical, but are known to express divergent behaviors, such as the alcohol-preferring P and alcohol-avoiding NP rat lines.

Methods

Animals

Twenty male alcohol-preferring rats (P rats: generation 76 and 79) and 8 male non-preferring rats (NP generation 76) were obtained from Indiana University at approximately 32 days old. Animals were handled 5 days per week for 4 weeks to habituate them to experimenters.

Groups: Pre-Recording Experience

P and NP rats were grouped into ethanol-naïve (e.g., Control: EN) and ethanol-experienced (EE) conditions. EE was comprised of 24-hour ethanol access for 3 days/week (e.g. Monday, Wednesday, and Friday) for 6 weeks in their home cages. Ethanol availability occurred in the

home cage immediately following lights out and consisted of three bottles, containing water, 15% (v/v) ethanol and 30% (v/v) ethanol for EE animals. EN animals (P rats, n=10; NP rats, n=4) had no previous alcohol experience, and only water during test sessions. Both groups had ad lib access to food throughout the experiment. Ethanol consumption was determined by weighing the bottles before and after the animal had access. We have previously validated this method of measuring ethanol consumption (Reno et al., 2015).

Ultrasonic Vocalization Recording

On the week immediately following ethanol or no ethanol experience, ultrasonic vocalizations (USVs) were recorded from each animal for 3 days/week (e.g. Monday, Wednesday, and Friday) over 4 weeks. On recording days, animals were weighed immediately following lights out and then placed into a recording environment that was identical to their home cage and used only for USV recording. Each animal had their assigned recording cage to prevent non-specific behaviors related to novelty or conspecific scents (Wohr et al., 2008). Animals were then transported to an adjacent testing room and USVs were recorded for 4 continuous hours. Immediately after the recording session animals were carted 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). Briefly, this program reads audio files and produces a spectrogram. The spectrogram is then searched for sound objects using MATLAB's *Image Processing Toolbox* (MathWorks, Inc. Natick, MA). A series of filters is then applied to separate background sounds from USVs. Finally, several measurements of interest are taken on each USV and stored for subsequent analysis.

Statistical Approach

Linear Mixed Model—We assessed differences in each USV characteristic as a function of rat line and alcohol-experience separately using a linear mixed model in R (R Core Team, 2015) using the package "lmerTest" (Kuznetsova, 2015). A series of models were applied to the data from each experimental group for each acoustic characteristic to examine if the goodness of fit of the model was improved by including time, selective breeding, previous ethanol experience, or an interaction of these factors. This is essentially a regression equation where the intercept term is allowed to vary for each rat. For example:

$$Y_{\text{Acoustic Characteristic}} = \beta_0 + \beta_{\text{Day}} X_{\text{Day}} + \beta_{\text{Alcohol}} X_{\text{Alcohol}} + \beta_{\text{PNP}} X_{\text{PNP}} + \beta_{\text{Alcohol*Day}} X_{\text{Alcohol*Day}} + \beta_{\text{PNP*Day}} + \beta_{\text{Alcohol*PNP}} + \beta_{\text{Alcohol*PNP*Day}} X_{\text{Alcohol*PNP*Day}} + W_{\text{Rat}}$$

where $Y_{\text{Acoustic Characteristic}}$ is the acoustic characteristic being modeled (e.g. mean frequency, duration, bandwidth, power), each predictor variable is represented by subscripts and W_{Rat} represents the random effect of individual rat. The coefficients (β) are estimated and assessed for significance. If found to be significant, then the coefficient's contribution to the goodness of fit of the model was assessed. When an interaction of two or more variables was found to be significant the package "LSMEANS" was used to conduct pairwise

comparisons (Lenth, 2016). This model was used in place of an ANOVA because it allows for the examination of raw data sets with unequal sample sizes (e.g. uneven emission of USVs).

Linear Discriminant Analysis—In addition, we subjected all four USV characteristics simultaneously to a linear discriminant analysis in the R package "MASS" (Venables, 2002) to determine if a linear combination of these data were capable of distinguishing groups of animals. Linear discriminant analysis is a multivariate statistical technique that allows the simultaneous consideration of multiple measures; in our case frequency, duration, bandwidth and power of each USV. A linear combination of the multivariate data produces a univariate value aimed at maximizing the separation of groups. For example, LDA can be used to determine whether USVs emitted by EN P rats differ from those emitted by EN NP 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. P rats and NP rats) without reference to time. The data are used in building the model, so it is possible that the model produced would be specific to those data and would not generalize to the population (e.g. P and NP rats) as a whole. As a way to ensure the generalizability of the model, half of the animals were used to train the model and half to test it. Naturally, when dividing the groups into training and testing subsets, certain combinations of animals within each subset will increase or decrease the ability of the model to separate the groups. Thus, to ensure an accurate assessment of this model, we bootstrapped the LDA 10,000 times and computed the percent of animals correctly assigned to their group[1]. The resulting distribution allowed us to estimate the average percent correct and standard error of this, which allowed us to compute 95% confidence intervals around this mean. 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 of 0.05.

Analysis of Variance—Mixed model ANOVAs were used to examine ethanol and water consumption as well as proportion of USV subtypes in P and NP rats.

Thus, the focus of this paper is on a rigorous statistical examination of USV acoustic characteristics in bidirectionally selected P/NP rat lines for 22–28 kHz (negative-affect) and 50–55 kHz (positive-affect) USVs. First, we examined each individual acoustic characteristic using a linear mixed model (LMM) to assess how the characteristics vary over time, between rat lines, and as the result of previous alcohol experience. Second, we examined the interactive effects of the acoustic characteristics taken as a whole and address whether these can be used to discriminate P rats from NP rats using linear discriminant analysis (LDA). Finally, we examined the more traditional measures of USV counts (e.g. %FM) and consummatory behavior (e.g. alcohol and water).

[1]To compute the percentage of animals correctly assigned to their groups by the LDA we took the following steps. First, we computed the average LDA value across all USVs emitted by each animal. Second, we computed the average of these animal by animal LDA averages at the group level. This yielded one average LDA for P rats and one average LDA for NP rats. Third, we computed the midpoint between these two means and used this as the threshold. Finally, we determined which animals were correctly classified into groups based on this threshold and computed that average number of animals correctly classified.

In each section of the analysis we focus on three main comparisons.

1. We compare ethanol-naïve P and NP rats to address issues of vulnerability to consume alcohol.
2. We compare ethanol-experienced P and NP rats to address differential effects across rat lines.
3. We compare ethanol-naïve with ethanol-experienced P rats to examine the effects of ethanol exposure within this alcohol-preferring rat line.

Results

USV Characteristics

The distribution of each characteristic of interest was examined and where appropriate log-transformed to improve normality. However, all figures display untransformed values.

Linear Mixed Model

LMMs were used to examine effects of selective breeding (e.g. P vs NP) and previous alcohol experience (e.g. ethanol-naïve: EN vs ethanol-experienced: EE) on the pattern of each characteristic over time. Any coefficients reaching our Bonferroni adjusted p-value of 0.0125 (0.05/4 USV characteristics) were examined for their impact on the model's goodness of fit. This was done by removing the factor with the significant coefficient from the model and comparing this reduced model with the full model. If the chi square value comparing these two models reached an alpha of 0.05 this indicated a significant difference in the goodness of fit. The factor was then reported as improving the fit of the model.

22–28 kHz USVs

Mean Frequency: A statistically significant three-way interaction between rat line, alcohol experience and day ($t(8307)=-22.19$, $p < 0.0001$) was seen to impact the model of Log10(Mean Frequency) of 22–28 kHz USVs (see Table 1 and Figure 1A). Removing this three-way interaction from the model resulted in a statistically significant reduction in the goodness of fit of the model ($\chi^2(2)=478.14$, $p < 0.0001$). Pairwise comparisons were performed to address the three specific comparisons of interest.

1. *Ethanol-Naive*: Mean frequency was significantly reduced ($p < .0125$) in NP relative to P rats on days 5 – 12
2. *Ethanol-Experienced*: Mean frequency was significantly reduced ($p < .0125$) in NP relative to P rats on days 1 – 5
3. *P*: No mean frequency differences emerged between EN and EE rats.

Duration: A statistically significant three-way interaction between rat line, alcohol experience and day ($t(8310)=-4.153$, $p < 0.0001$) was seen to impact the model of Log10(Duration) (see Table 1 and Figure 1B). Removing this three-way interaction from the model resulted in a statistically significant reduction in the goodness of fit of the model

($\chi^2(2)=17.4$, $p < 0.001$). Pairwise comparisons were performed to address the three specific comparisons of interest.

1. *Ethanol-Naive*: Duration was significantly reduced ($p < .0125$) in NP relative to P rats on days 2 – 12
2. *Ethanol-Experienced*: Duration was significantly reduced ($p < .0125$) in NP relative to P rats on all days
3. *P*: No duration differences emerged between EN and EE rats.

Bandwidth: A statistically significant two-way interaction of rat line and day ($t(8241)=2.66$, $p < 0.01$) was seen in log10(bandwidth), while the three-way interaction with EE approached significance ($t(8252)=-2.238$, $p = 0.025$; see Table 1 and Figure 1C). Removing the two-way interaction from the model reduced the model's goodness of fit ($\chi^2(2)=7.2$, $p < 0.05$). Pairwise comparisons were performed to address the three specific comparisons of interest.

1. *Ethanol-Naive*: Bandwidth was significantly reduced ($p < .0125$) in NP relative to P rats over days
2. *Ethanol-Experienced*: Bandwidth was significantly reduced ($p < .0125$) in NP relative to P rats over days
3. *P*: No bandwidth differences emerged between EN and EE rats.

Power: Power or decibel level varied widely and was related to day, rat line and alcohol experience as indicated by a statistically significant three-way interaction ($t(8307)=-8.85$, $p < 0.0001$). Removing the three-way interaction term from the model significantly reduced goodness of fit ($\chi^2(2)=77.96$, $p < 0.0001$; see Table 1 and Figure 1D). Despite the three-way interaction, none of the specific pairwise comparisons were significant.

1. *Ethanol-Naive*: No power differences emerged between NP and P rats
2. *Ethanol-Experienced*: No power differences emerged between NP and P rats
3. *P*: No power differences emerged between EN and EE rats.

Frequency-modulated 50–55 kHz FM USVs

Mean Frequency: An LMM of log10(mean frequency) of 50–55 kHz FM USVs revealed a statistically significant difference of rat line over days ($t(11510)= -5.89$, $p < 0.0001$; see Table 2 and Figure 2A), but no effect of previous alcohol exposure by day ($t(11520)= -1.4$, ns) or a three-way interaction ($t(11520)=1.44$, ns) was seen. Rat line by day significantly improved the goodness of fit of the model ($\chi^2(1)=40.87$, $p < 0.0001$). Pairwise comparisons were performed to address the three specific comparisons of interest.

1. *Ethanol-Naive*: Mean frequency was significantly reduced ($p < .0125$) in NP relative to P rats, but only on days 1 and 2
2. *Ethanol-Experienced*: Mean frequency was significantly reduced ($p < .0125$) in NP relative to P rats, but only on days 1 and 2

3. *P*: No mean frequency differences emerged between EN and EE rats.

Duration: A statistically significant effect of day ($t(11520) = -3.11$, $p < 0.01$) was seen in log10(duration) over days (see Table 2 and Figure 2B). Testing the influence of day on the goodness of fit revealed in a significant contribution to the model ($\chi^2(5)=32.6$, $p < 0.0001$). Pairwise comparisons were performed to address the three specific comparisons of interest.

1. *Ethanol-Naive*: No duration differences emerged between NP and P rats
2. *Ethanol-Experienced*: No duration differences emerged between NP and P rats
3. *P*: No duration differences emerged between EN and EE rats.

Bandwidth: No significant effect of rat line, previous alcohol experience, day or an interaction of these was seen for models of log10(bandwidth) (rat line by alcohol by day: $t(11130) = -1.22$, ns; rat line by day: $t(11510)=0.94$, ns; previous alcohol by day: $t(11060)=0.38$, ns; see Figure 2C). Thus, the results for the comparisons of interest were null.

1. *Ethanol-Naive*: No bandwidth differences emerged between NP and P rats
2. *Ethanol-Experienced*: No bandwidth differences emerged between NP and P rats
3. *P*: No bandwidth differences emerged between EN and EE rats.

Power: No significant effect of rat line, previous alcohol experience, day or an interaction of these was seen for models of power (rat line by alcohol by day: $t(11510)=0.46$, ns; rat line by day: $t(11510)=0.70$, ns; previous alcohol by day: $t(11510) = -0.63$, ns; see Table 2 and Figure 2D). Thus, the results for the comparisons of interest were null.

1. *Ethanol-Naive*: No power differences emerged between NP and P rats
2. *Ethanol-Experienced*: No power differences emerged between NP and P rats
3. *P*: No power differences emerged between EN and EE rats.

Brief Summary—The LMM results paint a clear story. First, three of the 22–28 kHz (negative-affect) USVs acoustic characteristics (frequency, duration and bandwidth) provided clear markers of alcohol vulnerability, as revealed by a comparison of ethanol-naïve P and NP rats. Only the frequency dimension of 50–55 kHz (positive-affect) USVs (and here only for days 1 and 2) reflected P and NP line differences. Second, three of the 22–28 kHz (negative-affect) USVs acoustic characteristics (frequency, duration and bandwidth), whereas only the frequency dimension of 50–55 kHz (positive-affect) USVs provided clear markers of the effects of alcohol exposure, as revealed by a comparison of ethanol-naïve P and NP rats. Finally, there was no evidence of USV differences within the P rat line as a function of alcohol-exposure (i.e., EN vs. EE) for 22–28 or 50–55 kHz USVs.

Linear Discriminant Analysis

22–28 kHz USVs: We examined the mean frequency (kilohertz; kHz), duration (milliseconds), bandwidth (kHz) and power (decibels) of the 22–28 kHz USVs simultaneously using a linear discriminant analysis.

1. *Ethanol-Naïve P vs. NP*: To determine if ethanol-naïve P and NP rats could be distinguished based solely on a linear combination of these acoustic characteristics, we estimated the linear discriminant from a set of "training" animals and used a separate set of animals to "test" the model. Because the performance of this model is based on which animals are included in the training and testing set, we bootstrapped this process 10,000 times to ensure an accurate assessment. Each iteration randomly selected half of the EN P rats and half of the EN NP rats to be used in training the model. The other half was used to test the model. The midpoint between the P and NP LDA values (testing group only) was determined and the percent of animals whose mean LDA value landed on the correct side of this midpoint was recorded. The vast majority (9790) of the 10,000 iterations resulted in perfect separation of P and NP animals, thus the computed 95% confidence interval was 100% - 100%. Because we were confident this model was performing well, we ran the LDA one last time including all of the EN P and NP rats to calculate the coefficients for each acoustic characteristic (see Table 3). This LDA applied to the full complement of data led to 100% separation of P from NP animals. This LDA was run on as a direct measure of the importance of each characteristic. Notice that for the EN data the importance from most to least was: duration, mean frequency, bandwidth and power.

2. *Ethanol-Experienced P vs. NP*: The same procedure was conducted to determine how alcohol experience influenced the distribution of USV acoustic characteristics. A little less than half (4866) of the 10,000 iterations resulted in perfect separation of P and NP rats. The mean percent correct for these EE animals was 87% and the 95% confidence interval around this mean was 57% – 100%, which does not include 50%. The representative coefficients were calculated including all of the EE P and NP rats (see Table 3). This LDA applied to the full complement of data led to 100% separation of P from NP animals. As with the EN data, this was run on the group standardized acoustic characteristics rendering the absolute value of the coefficients as a direct measure of the importance of each characteristic. For the EE data the importance from most to least was: duration, mean frequency, power, and bandwidth.

3. *P-Ethanol-Naïve vs. Ethanol-Experienced*: The same procedure was conducted to determine how alcohol experience influenced the distribution of USV acoustic characteristics in alcohol-preferring P rats. A non-significant separation of 47%[2] with 95% confidence interval of 20 – 70% was observed.

[2]Although the separation from the LDA applied to the training set is constrained to be above 50%, that constraint does not apply to the test set, as by chance values less than 50% are possible.

50–55 kHz USVs: We examined the mean frequency (kilohertz; kHz), duration (milliseconds), bandwidth (kHz) and power (decibels) of the 50–55 kHz USVs simultaneously using a linear discriminant analysis.

1. *Ethanol-Naïve P vs. NP*: We applied bootstrapped LDA modeling and found a mean percent correct separation of 36.5% with a 95% confidence interval of 14% – 71% which includes 50%.
2. *Ethanol-Experienced P vs. NP*: The same bootstrapping procedure was applied and we found a non-significant mean percent correct separation of 57.3% with 95% confidence interval of 14 – 86%.
3. *P-Ethanol-Naïve vs Ethanol-Experienced*: The same bootstrapping procedure was applied and we found a non-significant mean percent correct separation of 41% with 95% confidence interval of 20 – 60%.

Brief Summary: The LDA results mirror the LMM results and paint a clear story. First, whereas the 22–28 kHz (negative-affect) USVs acoustic characteristics provided clear markers of alcohol vulnerability, as revealed by a comparison of ethanol-naïve P and NP rats, the 50–55 kHz (positive-affect) USV characteristics did not. Second, whereas the 22–28 kHz (negative-affect) USVs acoustic characteristics provided clear markers of the effects of alcohol exposure, as revealed by a comparison of ethanol-experienced P and NP rats, the 50–55 kHz (positive-affect) USV characteristics did not. Importantly, frequency and duration were heavily weighted in both of these 22–28 kHz USV LDA analyses, in line with the LMM results. Finally, there was no evidence of USV differences within the P rat line as a function of alcohol-exposure for 22–28 or 50–55 kHz USVs.

Proportion 50–55 kHz Frequency-Modulated USVs—For completeness, we examined the number of positive and negative affect-related USVs emitted by examining the proportion of 50–55 kHz FM USVs (e.g. 50–55 kHz FM/(50–55 kHz FM + 22–28 kHz FM USVs). The ANOVA revealed only a significant effect of day ($F(11,264) = 2.83$, $p < 0.01$) (see Figure 3). The weak ANOVA results relative to the highly informative LMM and LDA results are a testament to the power of statistics that operate at the individual USV level as opposed to the aggregate.

Alcohol Consumption—As expected, the alcohol-preferring P rats consumed more ethanol than their non-preferring NP counterparts. An analysis of variance revealed a significant main effect of rat line ($F(1,12) = 46.016$, $p < 0.001$), and a significant interaction of rat line and day ($F(17,204) = 3.052$, $p < 0.001$; see Figure 4). Additionally, a Pearson's correlation revealed a statistically significant increase in alcohol intake over days for the P rats ($r = 0.266$, $p < 0.01$), but no trend for NP rats ($r = -0.0838$, n.s.).

Water Consumption—Water consumption during the 4-hour USV recording sessions was scaled to the animal's size (e.g. water intake (ml)/ body weight (g)) and examined for differences. A three way mixed model ANOVA revealed a main effect of rat line, such that NP animals ($M = 0.016$, $SEM = 0.001$) consumed more water than P animals ($M = 0.007$, $SEM = 0.001$; $F(1,24) = 21.8$, $p < 0.001$). An interaction between rat line and previous

alcohol experience revealed EN NP rats (M = 0.019, SEM = 0.002) consumed more than EE NP rats (M = 0.012, SEM = 0.002), while EN P (M = 0.007, SEM = 0.001) and EE P rats (M = 0.008, SEM = 0.001) animals did not differ ($F(1,24) = 5.24$, $p < 0.05$). Lastly, an interaction of P versus NP and day revealed NP rats water intake varied over days while P rats water intake was stable ($F(17,408) = 1.83$, $p < 0.05$; see Figure 5).

Discussion

Rodent USVs are accepted as a reflection of the animal's affective state (Brudzynski, 2009, Brudzynski, 2007, Burgdorf et al., 2008, Duvauchelle et al., in press). 22–28 kHz USVs are commonly associated with an animal's negative affective status and are initiated through ascending cholinergic pathways, frequency-modulated 50–55 kHz USVs are commonly associated with an animal's positive affective status and are initiated through the mesolimbic dopamine pathway (Ahrens et al., 2009, Brudzynski, 2007, Wright et al., 2012).

Preclinical research into alcohol use disorders has led to the development of divergent rat lines such as the alcohol-preferring (P) and non-preferring (NP) rats. P rats meet all of the criteria suggested for an animal model of alcoholism and displays multiple similarities with human alcoholics (McBride et al., 2014, Bell et al., 2012, Bell et al., 2016), thus providing an excellent translational animal model. Prior work from our laboratory showed that ethanol-naïve and ethanol-experienced P rats emitted unprovoked negative affect-related USVs, and that the two groups differed in the mean frequency of negative affect-related USVs, but not positive affect-related USV counts or acoustic properties (Reno et al., 2015).

The overriding aim of the current study was to explore the effects of rat line (P vs. NP) and ethanol-experience (naïve vs. experienced) on subsequent 22–28 kHz and 50–55 kHz USV profiles using advanced statistical techniques (linear mixed modeling and linear discriminant analysis; see Figure 6 for a schematic of LDA) that operate at the level of the individual USV emission, as opposed to some aggregate measure such as the mean. These approaches significantly increase the power of our statistical tests and allow us to explore effects of individual USV acoustic characteristics, such as frequency, duration, bandwidth and power, but importantly, interdependencies across these acoustic characteristics.

We address three main issues that organize the remainder of this Discussion. First, we examine whether USV acoustic characteristics reflect differences in vulnerability to consume alcohol by comparing ethanol-naïve P and NP rats. Second, we examine whether USV acoustic characteristics reflect differential effects of alcohol experience by comparing ethanol-experienced P and NP rats. Finally, we examine whether USV acoustic characteristics reflect differential effects of alcohol experience by comparing ethanol-naïve with ethanol-experienced rats just within the alcohol-preferring P rat line.

Vulnerability to Consume Alcohol: A Comparison of Ethanol-Naïve P and NP Rats

It is well established that P rats consume more alcohol than NP rats. This analysis addressed the question of whether this vulnerability to consume alcohol (if presented) is reflected in the USV profiles of alcohol-naïve P and NP rats. The results were clear. First, using linear discriminant analysis we showed that 22–28 kHz (negative-affect) USV profiles perfectly

separate ethanol-naïve P from ethanol-naïve NP rats, whereas 50–55 kHz FM (positive-affect) USV profiles do not. Second, we showed that 22–28 kHz USV frequency and duration information was most informative in separating these two USV profiles. Finally, these LDA analyses were strongly supported by linear mixed modeling at the individual USV acoustic characteristic level that showed 22–28 kHz differences between alcohol-naïve P and NP rats along frequency, duration and bandwidth, with no differences emerging for 50–55 kHz acoustic characteristics. Taken together, the results show that 22–28 kHz negative-affect, but not 50–55 kHz FM positive-affect USVs can be used to predict alcohol vulnerability in these animals. Given the strong link between 22–28 kHz USVs and mesolimbic acetylcholine, it is suggested that 22–28 kHz USVs can be considered as biomarkers for vulnerability to consume alcohol.

Effects of Alcohol Experience: A Comparison of Ethanol-Experienced P and NP Rats

This analysis addressed the question of whether alcohol-experience modulated the USV profile differences inherent across alcohol-naïve P and NP rats. Again, the results were clear. First, LDA showed that 22–28 kHz (negative-affect) USV profiles strongly separated ethanol-naïve P from ethanol-naïve NP rats, whereas 50–55 kHz FM (positive-affect) USV profiles do not. Second, 22–28 kHz USV frequency and duration information was most informative in separating these two USV profiles. Finally, these LDA analyses were strongly supported by linear mixed modeling at the individual USV acoustic characteristic level that showed 22–28 kHz differences between alcohol-naïve P and NP rats along frequency, duration and bandwidth, with no differences emerging for 50–55 kHz acoustic characteristics. These results suggest that 22–28 kHz negative-affect, but not 50–55 kHz FM positive-affect USVs continue to provide an important behavioral marker of differences between P and NP rats even once they are alcohol-experienced. This finding provides further support that 22–28 kHz USVs provide an important biomarker for alcohol vulnerability.

Effects of Alcohol Experience Within the Alcohol-Preferring P Rat Line

This analysis addressed the question of whether alcohol-experience changes the USV profile of alcohol-preferring P rats. The results were clear in showing that USV profiles did not differ within the P rat strain as a function of alcohol-experience (naïve vs. experienced) for either 22–28 kHz or 50–55 kHz USVs using LDA or LMM analyses. This finding suggests that P rat selective breeding leads to relatively concrete USV profiles that are unaffected by alcohol-experience.

USVs as a Biomarker

The neural pathways initiating 22–28 kHz negative and 50–55 kHz positive affective states and their corresponding vocalizations seem to have a homeostatic relationship. Activation of one inhibits the other when the activation is discrete; however, when the activation is long-lasting a homeostatic increase in the opposing state's pathway is seen. Given the relationship between the cholinergic and dopaminergic pathway, differences in one pathway may be reflected in the behavioral output of the other. Alcohol-preferring P rat have fewer dopaminergic neurons projecting from the VTA to the accumbens (Zhou et al., 1995) and lower baseline dopamine output compared to non-preferring NP rats (Murphy et al., 1982, Strother et al., 2005). In addition, multiple genetic indicators suggest that the P rats'

cholinergic system is hyperactive compared to the NP rat (Bell et al., 2016, McBride et al., 2013a, McBride et al., 2013b). Whether the result of selective breeding for alcohol preference in the alcohol-preferring P rat has been an overall increase in cholinergic activity or a decrease in dopaminergic activity or a combination of the two is a question for future research. For example, a decrease in the regulation of cholinergic activity by decreasing dopamine may allow for the hyperactivity seen in the cholinergic system. On the other hand, hyperactivity in the cholinergic system may be leading to a suppression of the dopaminergic system. Thus, the differences seen in the 22–28 kHz USV acoustic characteristics may be reflective of these differences in cholinergic and dopaminergic activity, and may offer an informative biomarker to be utilized in future research.

Implications for Human Alcohol Dependence

The implications of the present study on human alcohol dependence and other emotion-related disorders are many. This work suggests that emotional status affects vulnerability to alcohol abuse in rodents. In humans, this relationship between emotional status and drug or alcohol abuse has already been reported (Gizewski et al., 2013, Jaffe and Archer, 1987, McGue et al., 1997). For example, McGue and colleagues (1997) found that alcoholics scored higher on measures of negative emotionality than non-alcoholics. The present work also suggests that we can utilize naturalistic measures, such as an analysis of emotionality (e.g., electrophysiology), to determine significant associations with abuse potential. Along these lines, a number of laboratories are actively developing tools for classifying the emotional content of speech (Cohen et al., 2009, Choi et al., 2015), but to the best of our knowledge this research has yet to be applied to alcohol abuse. Given the strong relationship between negative-affect and alcohol use disorder in animals and humans, it is reasonable to propose that interventions (e.g., mindfulness-based interventions) that reduce the prevalence of negative affect might increase alcohol abstinence (i.e., prevent relapse). For instance, Pennebaker and colleagues show that expressive writing can reduce negative affect (Gallant and Lafreniere, 2003, Pennebaker, 2004, Pennebaker and Evans, 2014) potentially reducing vulnerability to alcohol abuse. These are exciting avenues of research that should continue to be pursued.

Acknowledgments

Research support: NIAAA T32 AA07471 (JMR), NIAAA013517 (CLD), AA015512, AA013522 (RLB), DA032457, MH077708, FA9550 (WTM), University of Texas Waggoner Center for Alcohol and Addiction Research

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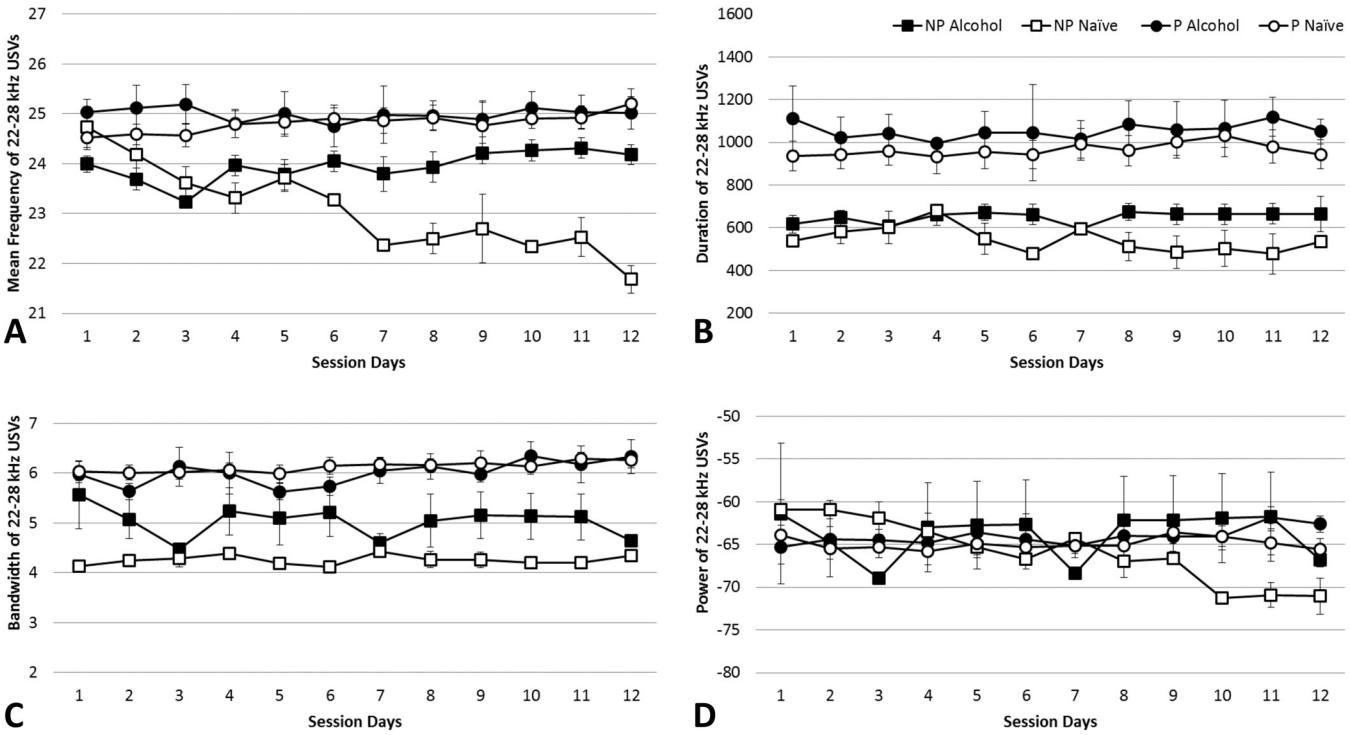

Figure 1. Rat Line and Previous Alcohol Experience Influence 22–28 kHz USV Acoustic Characteristics

Linear mixed models were used to model patterns in the USV acoustic characteristic data over time. A statistically significant three-way interaction between rat line, alcohol experience and day was seen to improve the fit of the model for **A)** mean frequency, **B)** duration and **D)** power. The model fit to bandwidth **(C)** revealed a statistically significant two-way interaction of rat line and day, while the three-way interaction with alcohol experience was trending towards significance ($p = 0.025$). Pairwise comparisons were conducted to identify which of the groups (e.g. NP Alcohol (NPA), NP Naïve (NPN), P Alcohol (PA), P Naïve (PN)) were significantly different. Mean frequency: NPA and PA days 1–5, NPN and PA days 3–12, NPN and PN days 5–12, NPN and NPA days 9–12, Duration: NPA and PA days 1–12, NPN and PA days 1–12, PN and NPA days 7–12, PN and NPN days 2–12, Power: NPN and PA days 11–12, Bandwidth: P and NP (not alcohol specific): days 1–12

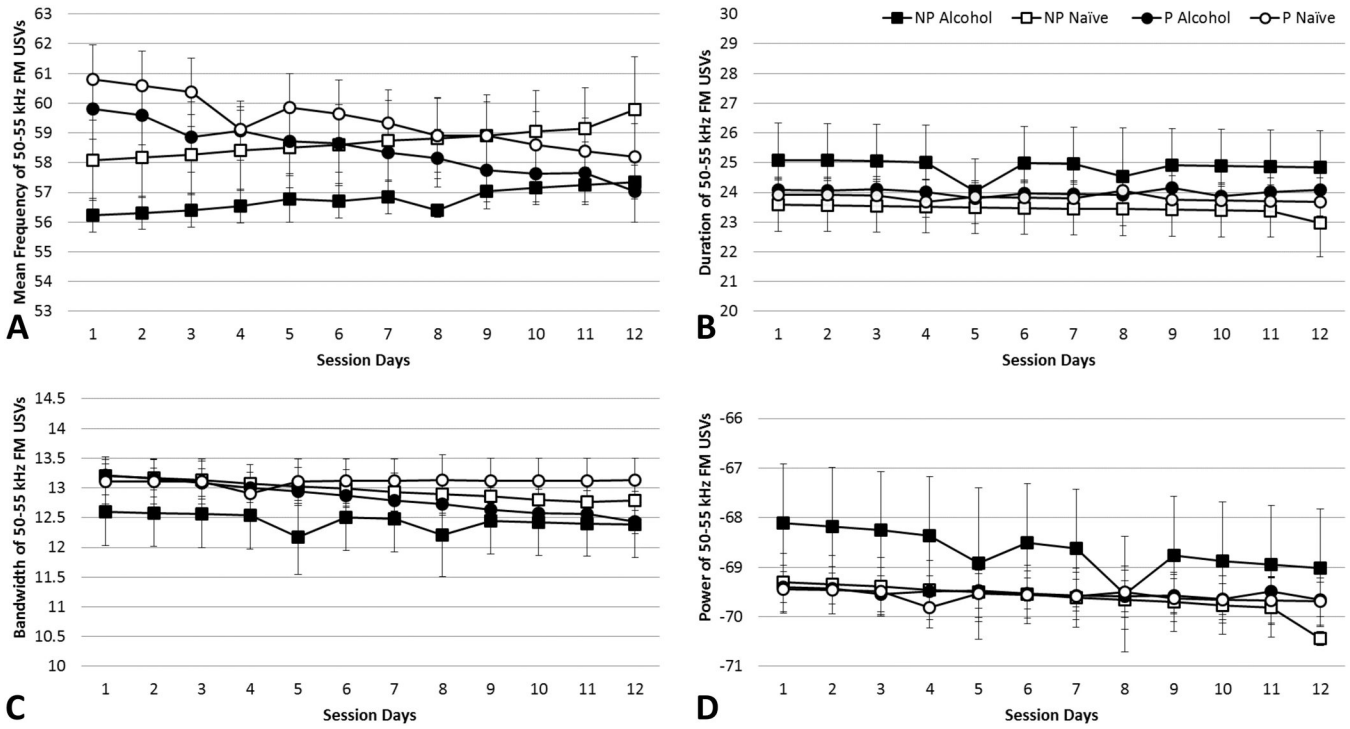

Figure 2. Rat Line and Previous Alcohol Experience Influence Certain 50–55 kHz FM USV Acoustic Characteristics

Modeling 50–55 kHz FM USV characteristics revealed an influence of rat line over days on **A)** mean frequency and a change over days on **B)**duration. Post hoc pairwise comparison on mean frequency revealed a significant difference between P and NP rats on day 1. No significant influences were detected on **C)**bandwidth or **D)**power.

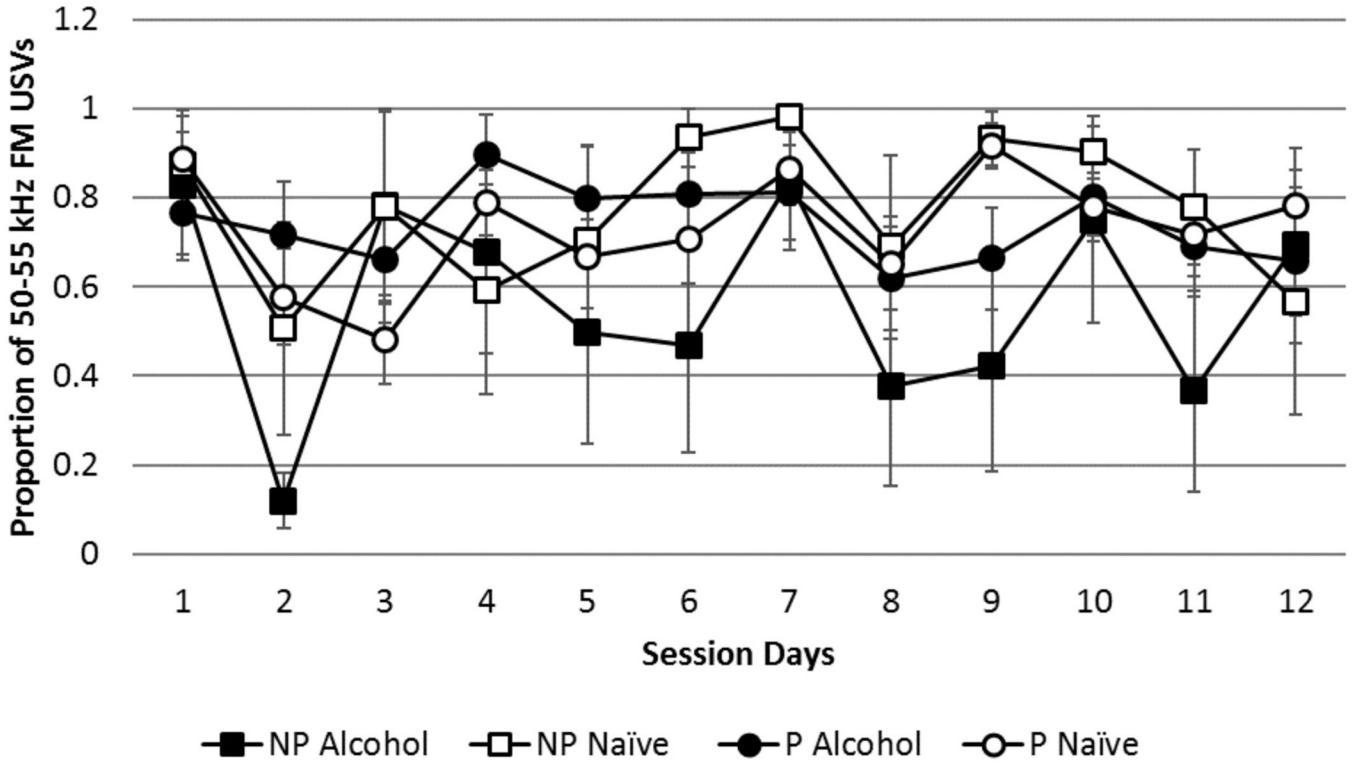

Figure 3. Marginal Ethanol Effect on Proportion 50–55 kHz FM USVs in NP but not P Rats

NP rats with previous ethanol experience displayed a marginally significant reduction in positive affect relative to negative affect. P rats with and without ethanol experience were similar in their affective profile.

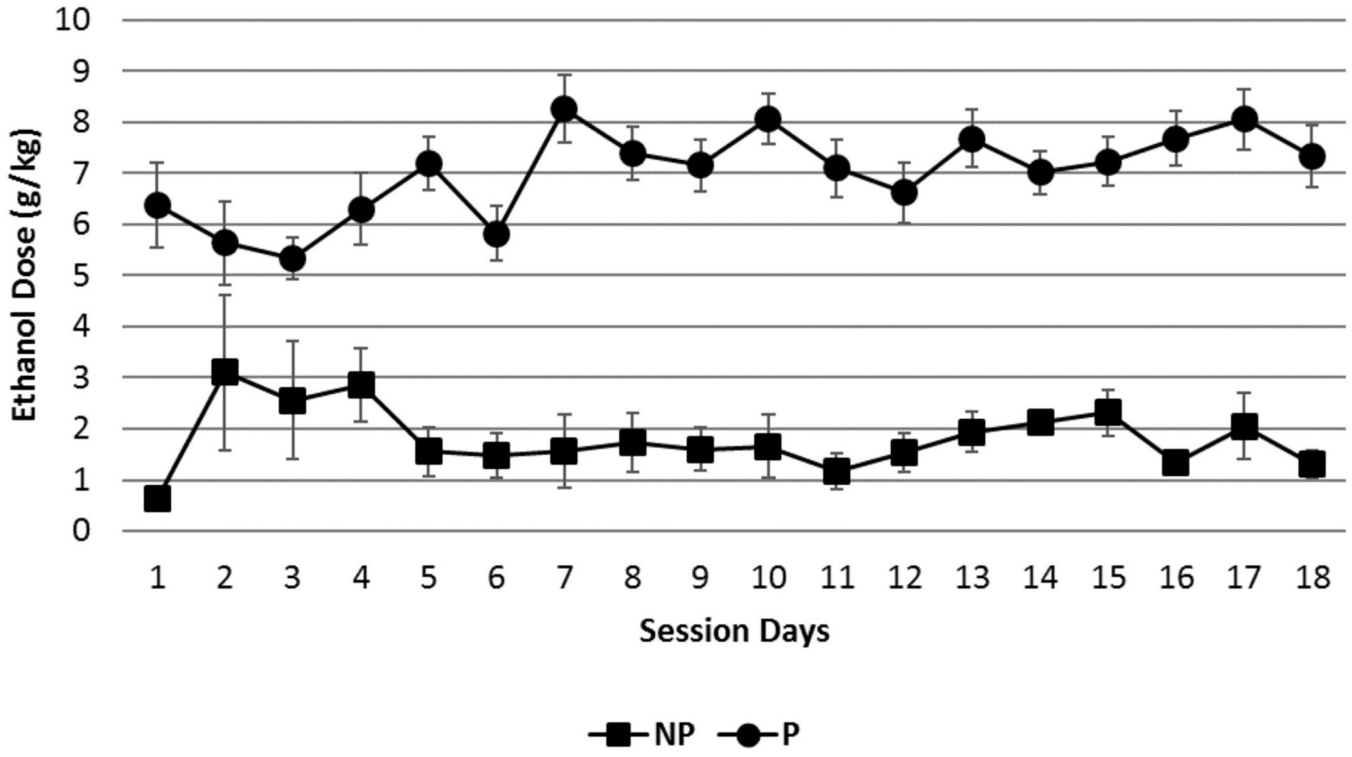

Figure 4. Ethanol Consumption of P and NP Rats

P rats consumed significantly more ethanol than non-preferring NP rats during six weeks of three bottle choice (15%, 30%, and H2O) 24-hr ethanol access sessions (3 days/week).

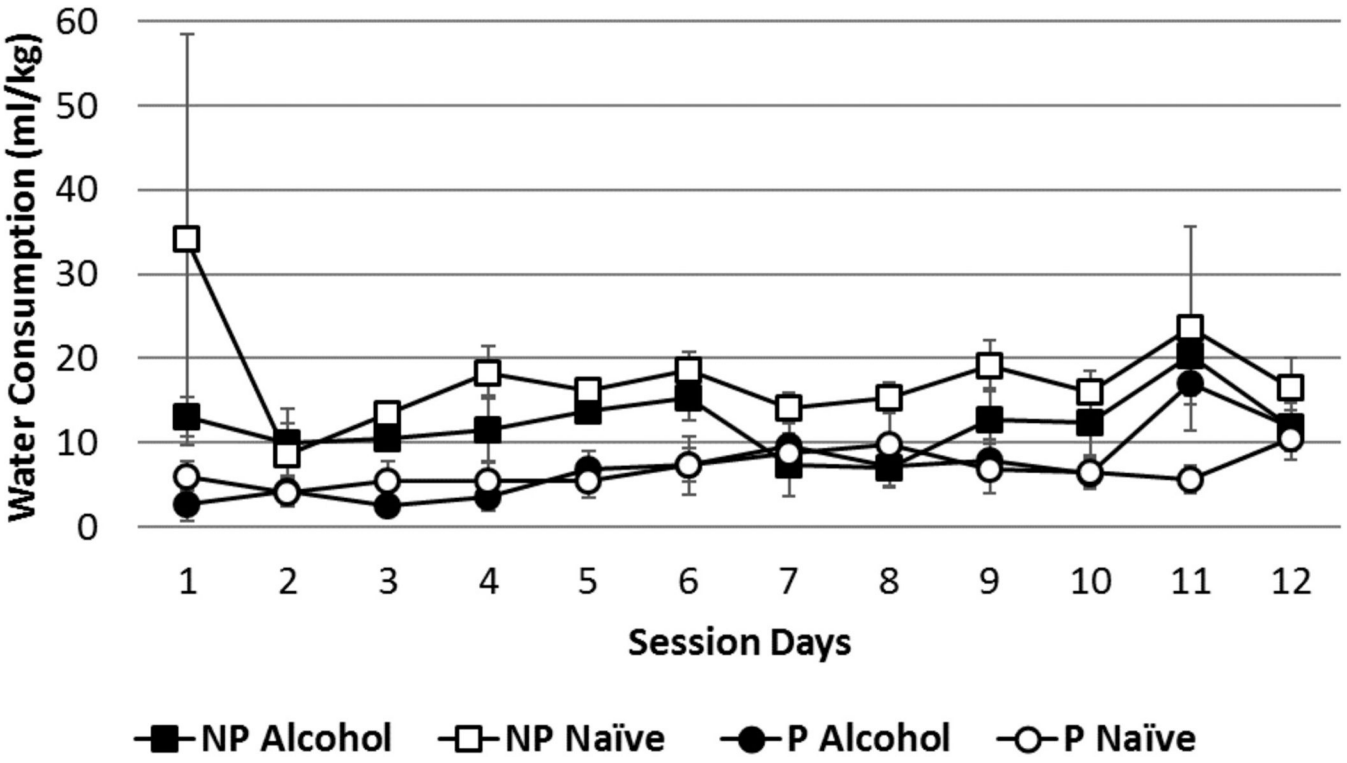

Figure 5. Water Consumption of P and NP Rats During USV Recording

Water consumption was greater overall in NP rats compared to P rats, though alcohol-experienced NP rats drank significantly less water than alcohol-naïve NP rats.

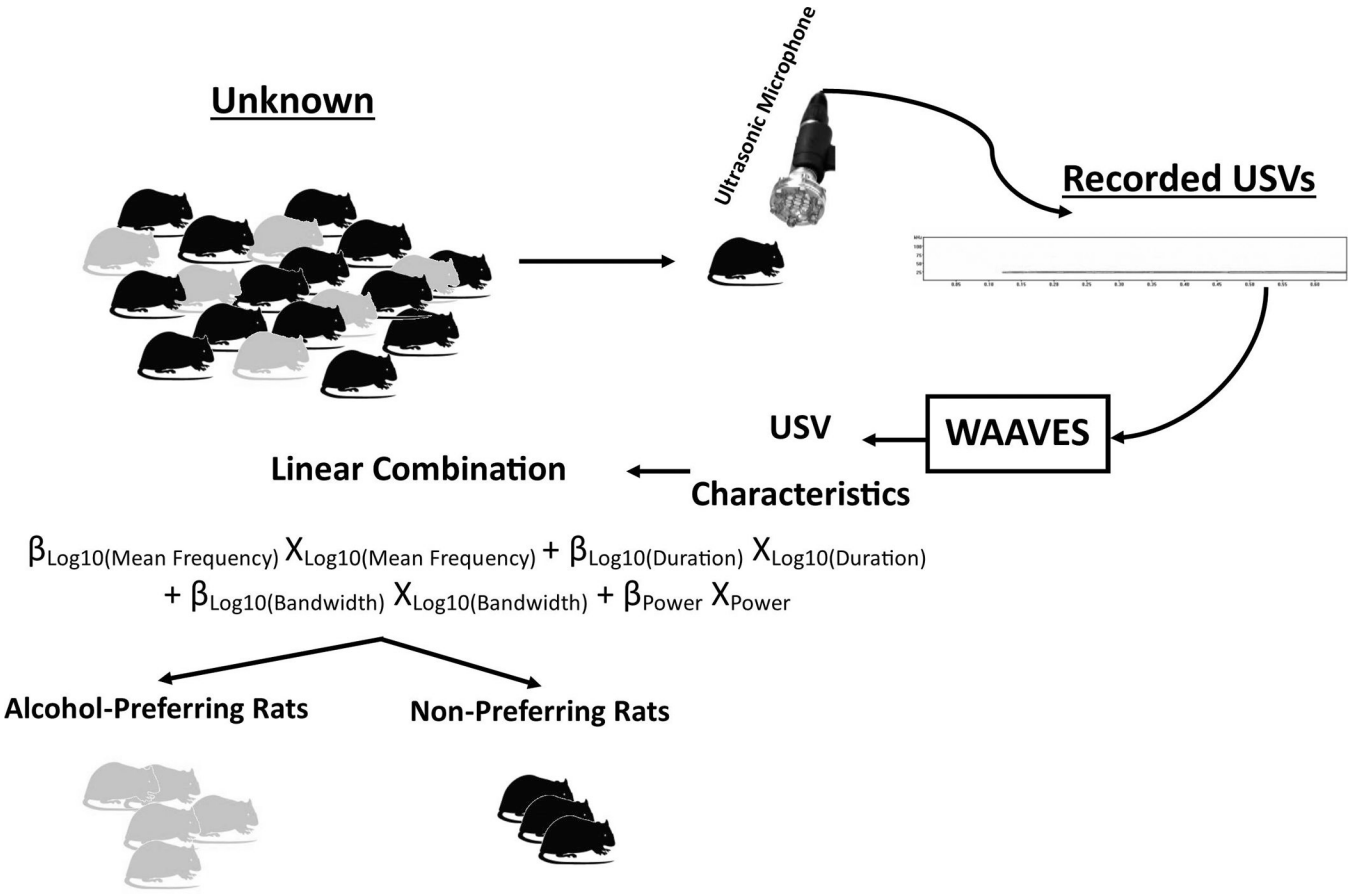

Figure 6. P and NP Rats Are Distinguishable by 22–28 kHz USV Acoustic Characteristics

Diagram depicts a process in which a mixed population of P and NP rats can be categorized into correct groups based solely on USV recordings of 22 kHz USVs. The classification procedure begins with USV recordings, followed by analyses of the vocalization recordings using the WAAVES program (Reno, et al 2013), and a resulting tabulation of 22–28 kHz USV acoustic characteristics (mean frequency, duration, bandwidth and power). Each set of USV acoustic characteristics is then subjected to a linear discriminant analysis to determine if alcohol-naïve P and NP rats can be distinguished based solely on a linear combination of these acoustic characteristics.

Table 1

Coefficient estimates for the Linear Mixed Models performed on 22–28 kHz USV acoustic characteristics.

Linear Mixed Models of 22–28 kHz USV Acoustic Characteristics								
$Y_{Acoustic\ Characterstic}$	β_0	β_{Day}	$\beta_{Alcohol}$	β_{PNP}	$\beta_{Alcohol*Day}$	$\beta_{PNP*Day}$	$\beta_{Alcohol*PNP}$	$\beta_{Alcohol*PNP*Day}$
Log_{10}(Mean Frequency)	1.39 ± 0.00617	−0.00175 ± 0.0000873	−0.0149 ± 0.00871	−0.000365 ± 0.00732	0.00214 ± 0.000101	0.0022 ± 0.000109	0.0285 ± 0.0104	−0.00288 *** ± 0.00013
Log_{10}(Duration)	2.81 ± 0.0519	−0.00583 ± 0.000964	−0.000351 ±0.0733	0.155 ± 0.0618	0.00601 ± 0.00112	0.00708 ± 0.0012	0.0304 ± 0.0875	−0.00595 *** ± 0.00143
Log_{10}(Bandwidth)	0.637 ± 0.0223	−0.00118 ± 0.000641	0.0636 ± 0.0314	0.137 ± 0.0269	0.000895 ± 0.000743	0.00212 ** ± 0.000798	−0.0544 ± 0.038	−0.00213 † ± 0.000951
Power	−60.3092 ± 2.2408	−0.4221 ± 0.0324	−4.7555 ± 3.1657	−5.2378 ± 2.6628	0.4992 ± 0.0375	0.4725 ± 0.0404	4.5906 ± 3.766	−0.4258 *** ± 0.0481

Rows denote acoustic characteristics and columns denote the b weights associated with each factor

Note: Coefficient estimates ± standard error,

** = $p < 0.01$,

*** = $p < 0.001$,

† = approaching significance

Table 2

Coefficient estimates for the Linear Mixed Models performed on the acoustic characteristics of the 50–55 kHz USVs.

Linear Mixed Models of 50–55 kHz FM USV Acoustic Characteristics								
$Y_{\text{Acoustic Characterstic}}$	β_0	β_{Day}	β_{Alcohol}	β_{PNP}	$\beta_{\text{Alcohol*Day}}$	$\beta_{\text{PNP*Day}}$	$\beta_{\text{Alcohol*PNP}}$	$\beta_{\text{Alcohol*PNP*Day}}$
Log_{10}(Mean Frequency)	1.76 ± 0.0116	−0.000494 ± 0.000178	−0.0109 ± 0.0165	0.0222 ± 0.0138	−0.000409 ± 0.000292	−0.0013 *** ± 0.00022	0.00236 ± 0.0196	0.000505 ± 0.000352
Log_{10}(Duration)	1.38 ± 0.0173	−0.00189 ** ± 0.000606	0.0184 ±0.0256	0.00409 ± 0.0212	0.00165 † ± 0.000994	0.000901 ± 0.000752	−0.0528 ± 0.0311	0.00137 ± 0.0012
Log_{10}(Bandwidth)	1.12 ± 0.0179	−0.000649 ± 0.000585	−0.0219 ± 0.0263	−0.00523 ± 0.0218	0.00036 ± 0.00096	0.000681 ± 0.000725	0.0277 ± 0.0319	−0.00141 ± 0.00116
Power	−69.3 ± 0.757	−0.022 ± 0.0138	1.22 ± 1.09	−0.149 ± 0.905	−0.0144 ± 0.0228	0.012 ± 0.0172	−1.17 ± 1.29	0.0127 ± 0.0274

Note: Coefficient estimates ± standard error, yellow = statistically significant at alpha = 0.0125, orange = approaching significance

Table 3

Coefficients for Linear Discriminant Analysis performed on all P and NP animals within the groups of alcohol-naïve and alcohol-experienced. Both LDAs resulted in 100% separation of the P and NP animals.

Linear Discriminant Analysis				
P or NP	$\beta_{Log10(Mean\ Frequency)}$	$\beta_{Log10(Duration)}$	$\beta_{Log10(Bandwidth)}$	β_{Power}
Alcohol-Naïve	0.604	0.757	0.387	−0.337
Alcohol-Experienced	0.754	0.922	−0.050	−0.478