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*Behav Brain Res.* 2016 April 1; 302: 228–236. doi:10.1016/j.bbr.2016.01.042.**Alcohol enhances unprovoked 22–28 kHz USVs and suppresses USV mean frequency in High Alcohol Drinking (HAD-1) male rats****Neha Thakore, PharmD<sup>a,c</sup>, James M. Reno, B.A.<sup>b,c</sup>, Rueben A. Gonzales, PhD<sup>a,c</sup>, Timothy Schallert, PhD<sup>b,c</sup>, Richard L. Bell, PhD<sup>d</sup>, W. Todd Maddox, PhD<sup>b</sup>, and Christine L. Duvauchelle, PhD<sup>a,c,\*</sup>**<sup>a</sup>The University of Texas at Austin, College of Pharmacy, Division of Pharmacology and Toxicology, 2409 University Avenue, Stop A1915, Austin, TX 78712, USA<sup>b</sup>The University of Texas at Austin, Department of Psychology, 108 E. Dean Keeton Avenue, Stop A8000, Austin, TX 78712, USA<sup>c</sup>Waggoner Center for Alcohol and Addiction Research, The University of Texas at Austin, 2500 Speedway, Stop A4800, Austin, TX 78712, USA<sup>d</sup>Institute of Psychiatric Research, Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN 46202, USA**Abstract**

Heightened emotional states increase impulsive behaviors such as excessive ethanol consumption in humans. Though positive and negative affective states in rodents can be monitored in real-time through ultrasonic vocalization (USV) emissions, few animal studies have focused on the role of emotional status as a stimulus for initial ethanol drinking. Our laboratory has recently developed reliable, high-speed analysis techniques to compile USV data during multiple-hour drinking sessions. Since High Alcohol Drinking (HAD-1) rats are selectively bred to voluntarily consume intoxicating levels of alcohol, we hypothesized that USVs emitted by HAD-1 rats would reveal unique emotional phenotypes predictive of alcohol intake and sensitive to alcohol experience. In this study, male HAD-1 rats had access to water, 15% and 30% EtOH or water only (i.e., Controls) during 8 weeks of daily 7-hr drinking-in-the-dark (DID) sessions. USVs, associated with both positive (i.e., 50–55 kHz frequency-modulated or FM) and negative (i.e., 22–28 kHz) emotional states, emitted during these daily DID sessions were examined. Findings showed basal 22–28 kHz USVs were emitted by both EtOH-Naïve (Control) and EtOH-experienced rats, alcohol experience enhanced 22–28 kHz USV emissions, and USV acoustic parameters (i.e., mean frequency in kHz) of both positive and negative USVs were significantly suppressed by chronic alcohol experience. These data suggest that negative affective status initiates and maintains excessive alcohol intake in selectively bred HAD-1 rats and support the notion that unprovoked

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emissions of negative affect-associated USVs (i.e., 22–28 kHz) predict vulnerability to excessive alcohol intake in distinct rodent models.

## Keywords

WAAVES; Drinking-in-the-dark; Emotional status; Negative affect; Excessive alcohol intake

## 1. Introduction

Emotional temperaments fuel impulsive behaviors and increase the risk of excessive ethanol drinking in humans [1–3]. For instance, there is ample evidence that alcoholics and heavy drinkers display enhanced impulsivity traits (i.e.; lack of premeditation, lack of focused attention, sensation seeking, negative urgency, positive urgency, and reward seeking) [1–3]. Furthermore, these traits are heritable, as children of parents with substance abuse disorders often externalize these behaviors even before the initiation of alcohol or substance abuse [4]. For instance, a clinical neurophysiological endophenotype for moderate dose alcohol effects and is seen in Family History Positive (FHP), for alcoholism, individuals, with or without alcohol experience, is increased latency and reduced amplitude of the P300 and N100 event-related potentials (ERPs) [5, 6]. Moreover, these ERPs are associated with maturation and degeneration of attentional processes across the lifespan [7]. Additionally, these endophenotypes are also markers for other addictions and associated impulsive behaviors [8, 9].

The tendency to act rashly when in heightened negative or positive emotional states are impulsivity characteristics termed “negative or positive urgency”, respectively, and are strongly predictive of alcohol-related problems [10, 11]. Negative urgency in particular is consistently correlated with severity of undesirable outcomes from alcohol [10, 12, 13]. Taken together, these findings indicate that impulsivity and emotionality play critical roles in the initiation, maintenance, and development of alcohol use disorders (AUDs).

Ultrasonic vocalizations (USVs) emitted by rats are considered to be a reflection of their real-time emotional state and are widely accepted as animal models of affect [14, 15]. Rodents emit USVs in the 50–55 kHz and 22–28 kHz ranges, which are reliably associated with positive and negative emotional states, respectively [15–17]. USVs have received increased attention in drug abuse studies because administration of cocaine [18], amphetamine [19] and drug-associated cues [20, 21] 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 [22] and alcohol-dependent rats in a state of withdrawal are more easily provoked to emit negative affect-associated 22–28 kHz USVs by mild aversive stimuli [23, 24]. Ascending mesolimbic cholinergic [25] and dopaminergic pathways [26] mediate production of 22–28 kHz and 50–55 kHz FM USVs, repeatedly, in correspondence with negative and positive emotional states [14, 27, 28]. Additionally, these mesolimbic pathways are activated during ethanol consumption [29, 30]. Therefore, we can achieve important insight into alcohol motivational processes by examining the relationship between alcohol experience and USV emission patterns.

Selective breeding for high alcohol intake in rats has produced a number of possible models for excessive alcohol intake and alcoholism [31–34]. Recently, our laboratory has recorded USVs from one of these models, the alcohol-preferring (P) rat. During a Drinking in the Dark (DID) experiment, we observed spontaneous 22–28 kHz USV emissions from both alcohol-naïve and alcohol-experienced P rats [24]. In the alcohol literature, 22–28 kHz USVs have been intentionally provoked by exposure to aversive air-puff stimuli in alcohol-dependent animals undergoing alcohol withdrawal [23, 35], confirming that negative emotional responses are easily aroused under these circumstances. However, unprovoked 22–28 kHz USVs observed in P rats suggests that negative affective temperament plays an important role in both initial and continued alcohol consumption. 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 [36]. The HAD-1 rats are not as well-characterized as the alcohol-preferring (P) rats but do meet most of the criteria set forth for a suitable animal model of alcoholism [37].

In order to characterize an emotional endophenotype of HAD-1 rats and understand how alcohol consumption influences that phenotype, we recorded ultrasonic vocalizations of HAD-1 rats in EtOH (three bottle choice of water, 15%, and 30% EtOH) and Control (water only) treatment groups across 8 weeks of DID sessions. We tested the hypothesis, which was derived from our previous work with P-rats, that a negative baseline affect (e.g., predominated by 22–28 kHz USV emissions) will reveal itself early on in both treatment groups, and will be further enhanced in the alcohol-experienced animals. Additionally, we performed in-depth examinations of the acoustic properties of 22–28 kHz and 50–55 kHz FM USVs, including mean frequency and duration, with the prediction that these parameters will be altered by alcohol experience. We previously reported that alcohol-experienced P rats show decreased mean frequency of 22–28 kHz USVs compared to alcohol-naïve P rats, while call duration was unaltered in both USV types [24]. Other studies have shown that mean frequency and duration of 22–28 kHz USVs were altered under different drug treatments, but 50 kHz FM USV acoustic patterns remained constant [27, 38, 39]. Taking all of these studies into account, we hypothesized that repeated and excessive alcohol consumption in HAD-1 rats will alter their USV profile with regard to both numerical counts and acoustic parameters.

## 2. Materials and Methods

### 2.1 Subjects

We received 24 male high-alcohol-drinking rats (HAD-1 generation = 63, 66) 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 1000) and were pair-housed in plastic cages (22 × 44 × 20 cm). Animals were handled daily for 4 weeks prior to the start of the experiment outside of the vivarium in a behavioral testing room distinguished by an olfactory cue (cinnamon vanilla scent). Animals were group-housed in wire-topped plastic cages (22 × 44 × 20 cm) until 1 week prior to the start of the experiment when they were pair-housed. Rats received food and water ad libitum throughout the entire

experiment. All of the procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee.

## 2.2 Procedures

**2.2.1 Apparatus**—DID sessions were conducted in the same behavioral testing room used during the handling phase (see above). The experimental chambers within the testing room 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. Although animals were housed in pairs in the vivarium, they were tested singly during DID sessions. Each animal was assigned to a specific test chamber to control for nonspecific USV emissions induced by novel environments and conspecific odors (Wohr et al., 2008).

**2.2.2 USV Recording**—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.

**2.2.3 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 [40]. 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. Some of the conditions specify USV acoustic parameters, such as frequency range and variation (e.g., in kHz), USV call duration and inter-call intervals (e.g., in milliseconds). Acoustic parameters and sound conditions were defined largely in accordance with existing USV literature [15, 28, 41], while certain settings (e.g. minimum call duration and inter-call intervals) were based on our experience with noise filtering during USV data collection. For these data 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.

**2.2.4 Acoustic parameters**—Acoustic parameter data, including frequency (kHz) and duration measures of each USV, are generated during the WAAVES tabulation process. Mean frequency was defined as the grand mean of frequencies determined at every half millisecond of each call. USV duration was simply the duration (ms) of each call.

**2.2.5 Validation Process for WAAVES Automation**—Subsets of USV data recorded during the DID procedure (fifty 1-min USV files for the 50–55 kHz USVs and fifty 10-min files for the 22–28 kHz USVs) were analyzed by research assistants blind to experimental conditions. These data sets were then analyzed using the WAAVES program to determine a

strong correspondence between WAAVES-generated USV counts and human-derived counts obtained through visual and auditory means.

**2.2.6 Drinking-in-the-Dark Sessions**—Drinking-in-the-Dark (DID) sessions commenced at the start of the dark cycle for all HAD-1 rats. Animals were housed under a reverse light/dark cycle (e.g., lights out at 1000) and weighed 5 days per week just after lights out. After weighing, animals were transported to the DID testing room. DID sessions, conducted in the dark with only red illumination, were 7 hours in duration and consisted of three 1-hour drinking intervals (e.g., “EtOH ON”) interspersed with two 2-hour water only intervals (e.g., “EtOH OFF”). During “ON” drinking intervals, rats had access to three sipper tubes (EtOH group: H<sub>2</sub>O, 15% ethanol, and 30% ethanol; Control group: three H<sub>2</sub>O tubes). During the “OFF” intervals, only water (one sipper tube) was available for all animals. Fluid intake was assessed gravimetrically after each drinking interval. USVs were recorded for the entire 7-hour session for each rat three days per week (first, third and fifth day of each week).

**2.2.7 Group Assignment**—Criterion for inclusion into the EtOH group required ethanol intake to reach at least 0.5 g/kg during all three 1-hr drinking intervals during daily DID sessions. Note that each DID session was defined as the 3 hours of total ethanol access, during the 7 hours of experimental access each day for 5 days per week. This value was derived from GC data showing that this dose produces intoxicating blood alcohol levels (Gonzales, personal communication). Of the animals originally assigned to the EtOH condition, approximately ½ met the ethanol intake criterion (n=8). Control animals (n=6) were given access to water only throughout the duration of the experiment. Data from a total of ten animals were excluded from data analyses, including nine originally assigned to the alcohol access group but did not meet the ethanol intake criterion and one from the Control group whose USV data files were corrupted.

**2.2.8 Validation of Alcohol Intake Measurements: Blood Alcohol Level Determination**—After the completion of the DID experiment, a subset (n=13) of ethanol-experienced HAD-1 rats were given 30 minutes of ethanol access and immediately anesthetized with isoflurane. The saphenous vein was punctured and blood was collected for determination of blood alcohol concentration. Triplicate 10 uL samples of blood were pipetted into glass vials containing 90 uL of saturated sodium chloride and sealed with a septum. Samples were heated for 1 hour at approximately 54 °C. Gas chromatography was conducted as previously described [30].

## 2.3 Statistical Analyses

**2.3.1 Daily EtOH and water intake**—EtOH intake (g/kg) across the 8 weeks of 7-hour DID sessions was analyzed in the EtOH group using a within-subjects repeated measures ANOVA. Total fluid intake (mL) across the 8 weeks of 7-hour DID sessions was compared between the EtOH and Control groups using 2 × 8 (group × week) mixed-design ANOVA.

**2.3.2 USV Counts, Acoustic Parameters and USV/EtOH Intake Correlation**—Mixed-design ANOVAs were used to compare weekly totals of 22–28 kHz and 50–55 kHz

FM USVs between the EtOH and Control groups across 8 weeks. Two-tailed t-tests were performed on weekly mean total 50–55 kHz FM and 22–28 kHz USV counts (e.g., USV totals of three 7-hour sessions/wk), 22–28 and 50–55 kHz FM USV mean frequencies (kHz) and durations (ms) during EtOH access (e.g., EtOH ON) and EtOH unavailability (e.g., EtOH OFF) intervals. Paired two-tailed t tests were performed to compare the proportion of 22–28 kHz and 50–55 kHz FM USV counts emitted by each rat during ON and OFF intervals in Weeks 1–2 and 3–8. Pearson's correlation was used to examine relationships between 22–28 kHz USVs and EtOH intake during EtOH ON and EtOH OFF intervals throughout the duration of the experiment.

**2.3.3 Validation of USV Counts and Ethanol Intake Measurements**—Pearson's correlation was used to examine the relationship between WAAVES tabulation and human-derived counts and the relationship between calculated intake levels of consumed EtOH (e.g., EtOH g/kg) and subsequent blood alcohol concentration (milligram percent) immediately after a 30-min EtOH access test.

### 3. Results

#### 3.1 Ethanol intake during DID sessions (EtOH ON Intervals – 3 hours total)

HAD-1 rats gradually acquired EtOH drinking to pharmacologically relevant levels. Within-subject repeated measures ANOVA showed significant changes in EtOH intake over the first two weeks of DID sessions ( $F(1, 7)=19.3$ ;  $p < 0.01$ ), indicating that the animals were acquiring ethanol intake during this time (see Fig. 1). There was no significant effect of time over Weeks 3–8 ( $F(5,35)=2.02$ ;  $p=0.1$ , n.s.), likely reflecting stabilization of ethanol intake.

#### 3.2 Total fluid intake during DID (7-h session)

EtOH and Control HAD-1 rats drank comparable amounts of fluid over the course of 8 weeks of DID sessions. A comparison of the total amount (mLs) of fluid consumption (e.g., EtOH and/or H<sub>2</sub>O) during each week of 7-hr DID sessions between EtOH and Control groups was performed using a 2 group  $\times$  8 week mixed design ANOVA. Similar levels of consumption between groups ( $F(1,12)=0.378$ ;  $p=0.50$ , n.s.) were observed, but a significant week effect ( $F(7,84)=11.08$ ;  $p<0.001$ ) emerged. This effect was likely the result of both EtOH and Control group drinking activity, which show variable patterns of fluid intake, including increasing fluid intake levels over time (see Fig. 2). Indeed, within-subject ANOVAs of fluid intake show significant week effects for both the Control group ( $F(7, 35)=2.285$ ;  $p<0.05$ ) and the EtOH group ( $F(7,49)=2.203$ ;  $p< 0.001$ ).

#### 3.3 USV Counts

**3.3.1 EtOH vs. Control and 22–28 kHz vs. 50–55 kHz FM USVs**—A mixed-design ANOVA conducted on the weekly total 22–28 kHz USVs emitted during DID sessions in Weeks 1–2 showed no significant differences between the EtOH and Control groups ( $F(1,12)=0.101$ ;  $p=0.756$ , n.s.). However, the EtOH group emitted significantly more 22–28 kHz USVs during Weeks 3–8 ( $F(1,60)=8.4$ ;  $p<0.05$ , see Fig. 3). With regard to 50–55 kHz FM USVs, no differences between groups were revealed during either Weeks 1–2 ( $F(1,12)=0.446$ ;  $p=0.5$ ) or Weeks 3–8 ( $F(1,60)=1.7$ ;  $p=0.211$ ) (see Fig. 4).

Within-subject analyses comparing 22–28 and 50–55 kHz FM USVs during EtOH ON and OFF intervals showed that the EtOH group emitted significantly more 22–28 kHz than 50–55 kHz FM USVs during EtOH OFF intervals ( $t(7)=2.365$ ;  $p<0.05$ ) and marginally more 22–28 kHz USVs during EtOH ON Intervals ( $t(7)=2.365$ ;  $p=0.06$ ) during Weeks 3–8. During DID sessions in Weeks 1–2, 22–28 kHz and 50–55 kHz FM USV counts did not significantly differ during EtOH OFF ( $t(7)=2.365$ ;  $p=0.349$ , n.s.) or EtOH ON intervals ( $t(7)=2.365$ ;  $p=0.585$ , n.s.; see Figs 5A and B). No significant differences in proportion of call types were evident for the Control group during the EtOH OFF ( $t(5)=2.571$ ;  $p=0.287$ , n.s.) or EtOH ON ( $t(5)=2.571$ ;  $p=0.182$ , n.s.) periods during Weeks 1–2. The same held true for the EtOH OFF ( $t(5)=2.571$ ;  $p=0.228$ , n.s.) or EtOH ON ( $t(5)=2.571$ ;  $p=0.571$ , n.s.) periods during Weeks 3–8.

### 3.4 USV Acoustic Patterns: Mean frequency

Compared to Controls, the EtOH group showed significant suppression in USV mean frequency in both 22–28 kHz ( $t(12)=2.179$ ;  $p<0.05$ ) and 50–55 kHz FM USVs ( $t(12)=2.179$ ;  $p<0.05$ ) emitted during EtOH ON intervals. This was not the case for either 22–28 kHz ( $t(12)=2.179$ ;  $p=0.128$ , n.s.) or 50–55 kHz USVs ( $t(12)=2.179$ ;  $p=0.179$ , n.s.) during EtOH OFF intervals (see Figs 6A and 6B).

### 3.5 USV Acoustic Patterns – Mean Duration

Mean duration of 22–28 kHz USVs did not differ significantly during either the EtOH ON ( $t(12)=2.179$ ;  $p=0.226$ , n.s.) or EtOH OFF periods ( $t(12)=2.179$ ;  $p=0.201$ , n.s.). Mean duration of 50–55 kHz USVs did not differ significantly during either the EtOH ON ( $t(12)=2.179$ ;  $p=0.074$ , n.s.) or EtOH OFF periods ( $t(12)=2.179$ ;  $p=0.631$ , n.s.).

### 3.6 Correlational analyses of 22–28 kHz USV Counts and EtOH intake

HAD-1 rats in the EtOH Condition ( $n=8$ ) displayed a significant positive linear relationship between EtOH intake and 22–28 kHz USV counts during EtOH OFF ( $r = 0.87$ ;  $p < 0.01$ ), but not during EtOH ON intervals ( $r=-0.11$ ;  $p=0.8$ , n.s., see Figs 7A and B).

### 3.7 Validation Tests: Correspondence Between WAAVES-Generated and Manual USV Assessments

WAAVES-automated analysis and manual human analysis were highly correlated for both 22–28 kHz USVs ( $r(48) = 0.99$ ;  $p < 0.001$ ; Fig. 7A) and 50–55 kHz FM USVs ( $r(48) = 0.99$ ;  $p < 0.001$ ; Fig. 7B).

### 3.8 Validation Tests: Blood alcohol concentration (BAC) and EtOH intake (g/kg)

Pearson's correlation was used to examine the relationship between ethanol consumption (grams of ethanol per kilogram of body weight) and blood alcohol concentration (milligram percent) after a 30-minute alcohol access test interval. The results showed that ethanol consumption levels were significantly correlated with blood alcohol concentrations (BACs) (mg%,  $r(11)=0.69$ ;  $p<0.01$ , see Fig. 8).

## 4. Discussion

This study revealed that HAD-1 rats emit unprovoked 22–28 kHz USVs, which are increased by alcohol experience. Though this finding is consistent with our recent report on the selectively bred alcohol-preferring P rat [24], spontaneous 22–28 kHz USV emissions have not been reported in any other rat lines. In addition, the mean frequency of positive affect USVs (approx. 65–70 kHz) is significantly higher than in our previous findings in P [19] and Sprague-Dawley rats [13–16] and may be unique to the HAD-1 rat line. HAD-1 rats in the EtOH condition also maintained a higher proportion of negative (e.g., 22–28 kHz) compared to positive affect (e.g., 50–55 kHz FM) USVs throughout the DID sessions, including both EtOH ON and OFF intervals. Additionally, we also found significant differences in USV acoustic parameters in both the 22–28 kHz and 50–55 kHz FM ranges between groups. These findings strengthen the hypothesis that chronic alcohol intake has direct effects on neural pathways and/or affect that mediate USV emissions in the HAD-1 rat line.

We recently reported ethanol consumption in alcohol-preferring P rats that was lower than previous reports [24]. We attributed lower ethanol consumption levels to the extended pre-experimental handling procedures (e.g., 4 weeks of daily handling sessions) used to decrease anxiogenic and/or negative emotional responses to human touch since other DID studies reporting higher ethanol intake levels do not report a handling phase in their experiments [31–34]. Similarly, the current study showed average ethanol intake at 2.9 ( $\pm$  0.06) g/kg/session, which was noticeably lower than others have reported for HAD-1 rats [42]. However, the latter study measured 24-hour periods of free-choice access, whereas the present study measured three 1-hour periods of free-choice access per day. In addition, a large number of HAD-1 rats (9 out of 17) did not reach the ethanol consumption criterion (e.g., at least 0.5 mg/kg/1 hr during all three EtOH ON intervals in every DID session). In a previous behavioral study, specific deficits in avoidance responding in HAD rats led the authors to speculate that excessive anxiety may lead to high alcohol consumption in the HAD line [43]. If so, it is conceivable that decreased anxiogenic status could reduce alcohol consumption after extended handling procedures. This sequence of events would also support the notion that negative emotional states, such as anxiety contribute to excessive alcohol consumption in HAD-1 rats, though other as-of-yet unidentified traits of the HAD-1 line may contribute to these behaviors.

22–28 kHz USVs are initiated by activity of the ascending mesolimbic cholinergic pathway whereas 50–55 kHz FM USVs are initiated by activation of the mesolimbic dopaminergic pathway [14, 27, 28]. Voluntary ethanol intake activates components of both pathways, increasing acetylcholine (ACh) levels in the VTA and dopamine (DA) levels in the NAcc [29]. Indeed, we found that the EtOH group emitted significantly more 22–28 kHz USVs compared to Controls but that 50–55 kHz FM USV counts were comparable between both groups, consistent with our previous report on the alcohol-preferring P rat [24].

When considering USV counts during EtOH ON and OFF intervals, after EtOH drinking acquisition (e.g., during Weeks 3–8), we found that 22–28 kHz USVs were proportionally greater than 50–55 kHz FM USVs during both intervals. In addition, correlational analyses



examining relationships between EtOH intake and 22–28 kHz USV counts during EtOH ON and OFF intervals throughout the entire DID experiment (Weeks 1–8) showed a significant positive linear relationship between alcohol intake and 22–28 kHz USV emissions during EtOH OFF, but not during EtOH ON intervals. As such, these data indicate that negative USVs are not a consequence of EtOH drinking, but that the absence of EtOH further increases negative affective status in an alcohol experience-dependent manner.

Yet, both positive (e.g., 50–55 kHz FM) and negative affect (22–28 kHz) USVs emitted by the EtOH group were significantly lower in frequency (e.g., kHz) compared to Controls. It should be noted that HAD-1 rats display higher ethanol-induced DA efflux in the nucleus accumbens than LAD-1 rats [44]. Moreover, HAD rats have lower tissue levels of DA, DOPAC, and HVA in the nucleus accumbens and caudate putamen than their LAD counterparts [45]. Regarding the present findings, USV mean frequency increased in the Control group across the 8-week DID experiment, with 50–55 kHz USV mean frequency starting at 64.4 ( $\pm$  1.1 SEM) at Week 1 to 69.8 kHz ( $\pm$  1.9 SEM) at Week 8. The increase in USV mean frequency was suppressed in the EtOH group, starting at 63.1 ( $\pm$  1.5 SEM) and ending at 66.4 ( $\pm$  1.0 SEM) over the same time period. Although there was a slight increase in 22–28 kHz USV mean frequency in the Control group over time; 25.5 ( $\pm$  0.72 SEM) to 26.2 kHz ( $\pm$  0.9 SEM), the EtOH group showed a decrease in mean frequency over the 8 week experiment, going from 25.1 ( $\pm$  0.4 SEM) to 24.3 kHz ( $\pm$  0.5 SEM). Our findings of a significant shift in USV mean frequency after prolonged EtOH experience suggests that USV mean frequency is a more sensitive measure of ethanol-induced neural adaptations to cholinergic and dopaminergic pathways than USV counts.

There is extensive human literature linking emotional states, anxiety and impulsivity with alcohol-related problems [1–4, 10–13]. One study showed that individuals with anxiety disorders make rash decisions in order to alleviate the heightened distress they experience from their symptoms [46]. For example, negative and positive urgency are impulsivity traits defined as the tendency to act rashly while in heightened emotional states [10, 11]. Negative urgency, a heightened negative emotional state, in particular is significantly correlated with severe anxiety symptoms and is a strong predictor of alcoholism [10, 12, 13].

Similar to human alcoholics and heavy drinkers [47–50], HAD-1 rats score significantly higher on measures of impulsivity and risk-taking than their low alcohol drinking (LAD-1) counterparts [51]. In addition, our current findings show that HAD-1 rats possess a unique emotional phenotype comprised of an unusually high proportion of negative affect USVs that is further enhanced by alcohol consumption. These findings are reminiscent of human behavioral cycles wherein heightened impulsivity traits such as negative urgency lead to high levels of alcohol consumption, which in turn exacerbate behavioral expression of impulsivity traits [52]. Taken together, we suggest that impulsivity and emotionality play a critical role in the initiation and maintenance of alcohol drinking in the HAD-1 rat line. However, it is possible that these unique features may be due to other changes in this rat line that have not yet been identified.

The human literature has provided robust evidence that negative emotional states initiate and maintain alcohol-drinking behavior [53–56]. In traditional rat lines, 22–28 kHz USVs are

emitted in response to a number of negative stimuli, such as fear, illness, or pain [27, 28, 57]. In the absence of any of these conditions, it is possible that the unprovoked 22–28 kHz USVs observed in the selectively-bred HAD-1, of the present study, and P, in our previous study, rats [24] reflect a heightened negative affective state that facilitates alcohol motivation. However, it is important to note that baseline negative affect in HAD-1 rats is not likely due to depression, as shown by an animal model of depression (forced swim test). According to this study, HAD-1 and LAD-1 rats showed no difference in time spent immobile during a forced swim test, indicating that there is no functional relationship between high alcohol drinking and susceptibility to behavioral despair or depression, at least for this animal model [58].

The HAD-1 rat line meets most of the criteria proposed for an animal model of alcoholism, but has not yet been studied as extensively as the alcohol-preferring P rat, which meets all of the criteria proposed for an animal model of alcoholism [31, 32, 34]. Through in depth examinations of 22–28 kHz and 50–55 kHz FM USV profiles we have shown that HAD-1 rats exhibit a unique emotional phenotype dominated by negative emotional responses/USVs that are enhanced by binge alcohol drinking. Findings from the current study are consistent with the notion that this emotional profile contributes to excessive alcohol drinking vulnerability and that the HAD-1 rat line may closely model excessive alcohol consumption in alcohol users with high levels of both impulsivity and/or negative urgency traits.

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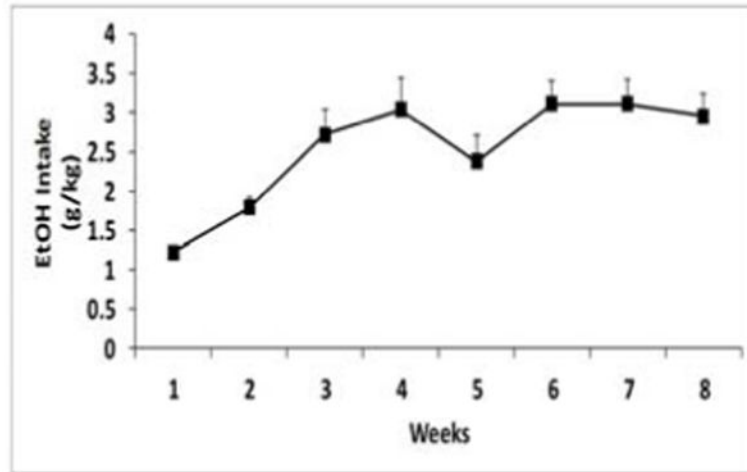
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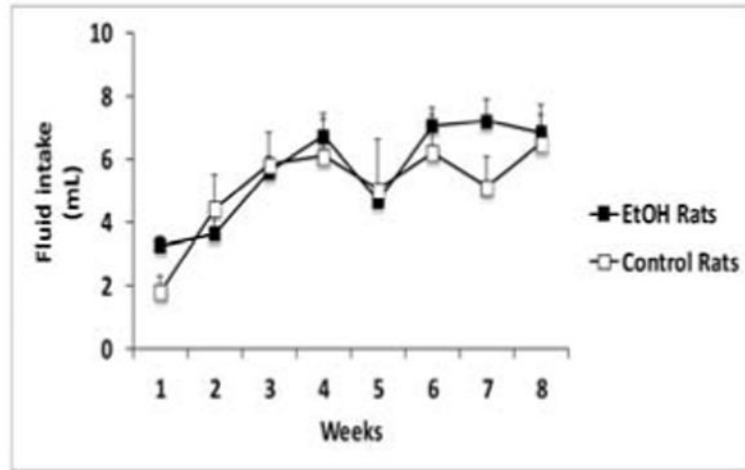
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**Highlights**

- Alcohol-naïve and alcohol-experienced HAD-1 rats spontaneously emit 22–28 kHz USVs
- A significant positive linear relationship between 22–28 kHz USV counts and alcohol intake is observed in alcohol-experienced HAD-1 rats in the absence of alcohol availability
- 22–28 kHz USVs are proportionally greater than 50–55 kHz FM USVs in alcohol-experienced HAD-1 rats
- 22–28 and 50–55 kHz USV mean frequency is suppressed in alcohol-experienced compared to alcohol-naïve HAD-1 rats

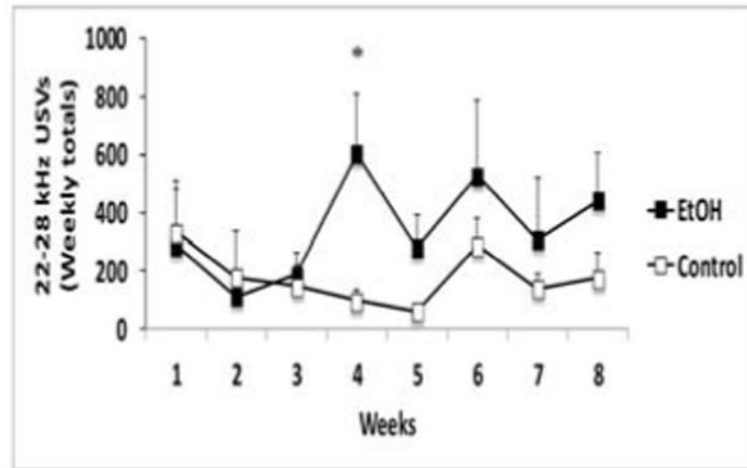


**Fig. 1.** Weekly EtOH intake during DID sessions. The overall mean intake across 8 weeks of DID sessions was 2.9 g/kg ( $\pm 0.06$ ; EtOH group,  $n=8$ ).

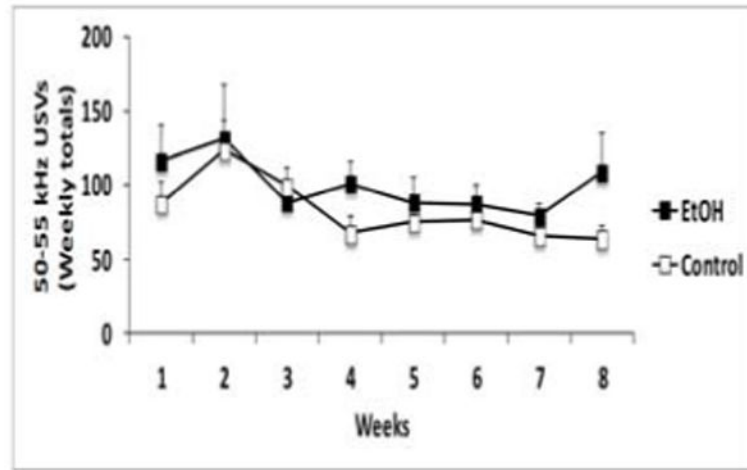


**Fig. 2.** Total fluid intake during DID sessions (mL; mean  $\pm$  SEM). Total fluid intake was comparable between EtOH and Control groups, and there was a significant Week effect that is likely the result of increasing fluid intake levels over time in both groups ( $p < 0.001$ ).



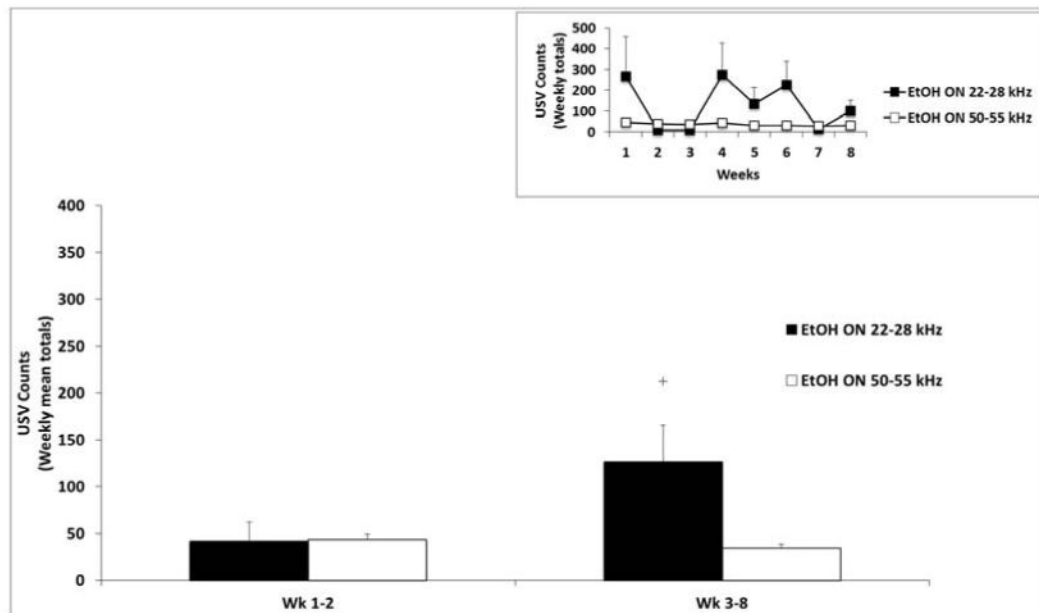


**Fig. 3.** 22–28 kHz USV counts in EtOH and Controls during (DID) sessions (weekly total  $\pm$  SEM). The EtOH group ( $n=8$ ) spontaneously emitted significantly more 22–28 kHz USVs than Controls ( $n=6$ ) during Weeks 3–8 ( $p < 0.05$ ), but not weeks 1–2. Post hoc tests showed significant differences between EtOH and Controls during Week 4 (\* =  $p < 0.05$ ).



**Fig. 4.** 50–55 kHz USV counts in EtOH and Controls during (DID) sessions (weekly total  $\pm$  SEM). 50–55 kHz FM USV counts did not vary significantly between EtOH and Control groups.

(A)



(B)

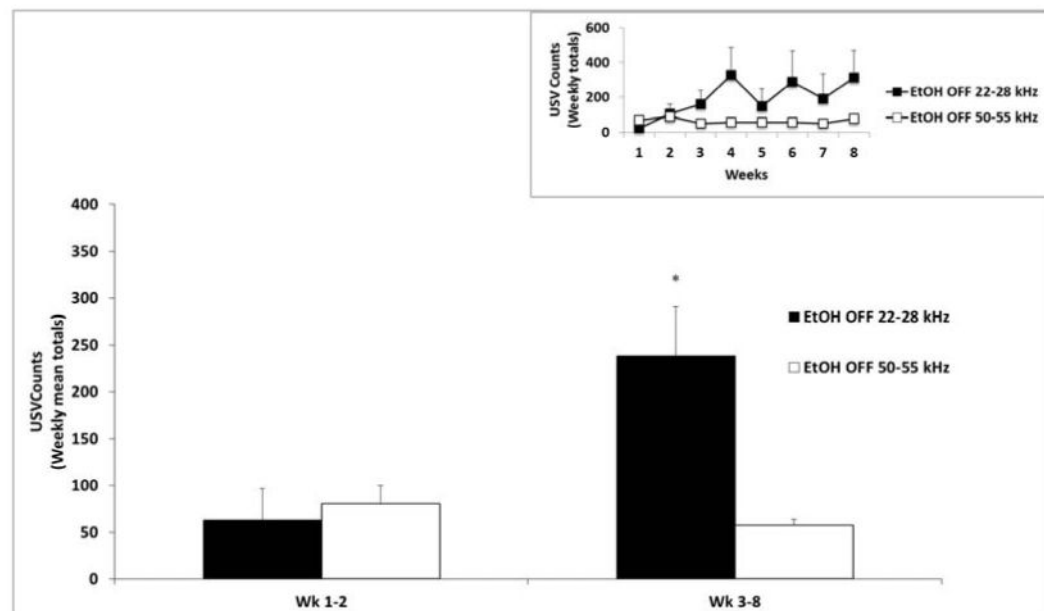


Fig. 5.

22–28 kHz and 50–55 kHz FM USV counts during EtOH ON and OFF intervals: Weeks 1–2 and Weeks 3–8 (mean  $\pm$  SEM). **A.** HAD-1 rats in the EtOH group emitted significantly more 22–28 kHz USVs than 50–55 kHz FM USVs during Weeks 3–8 during ethanol access (+ =  $p$  @ 0.06; marginal) *Inset:* Weekly USV totals during EtOH ON intervals (3-hour/DID session, 3 days/week). **B.** HAD-1 rats in the EtOH group emitted significantly more 22–28 kHz USVs than 50–55 kHz FM USVs during Weeks 3–8 during periods of ethanol absence

(\* =  $p < 0.05$ ). *Inset*: Weekly USV totals during “EtOH OFF” intervals (4-hour/DID session, 3 days/week).

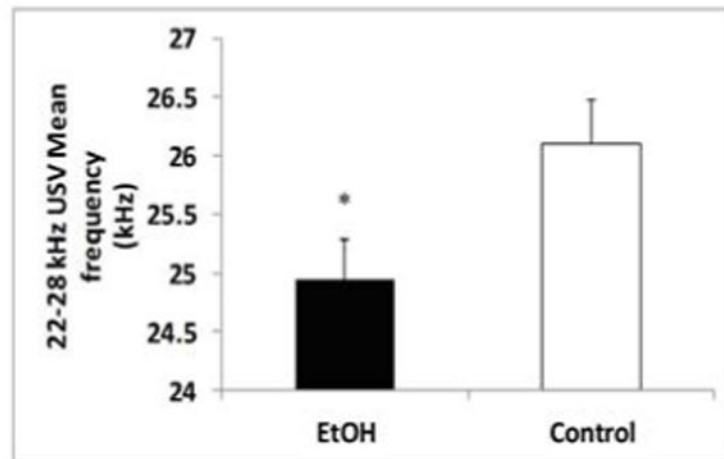
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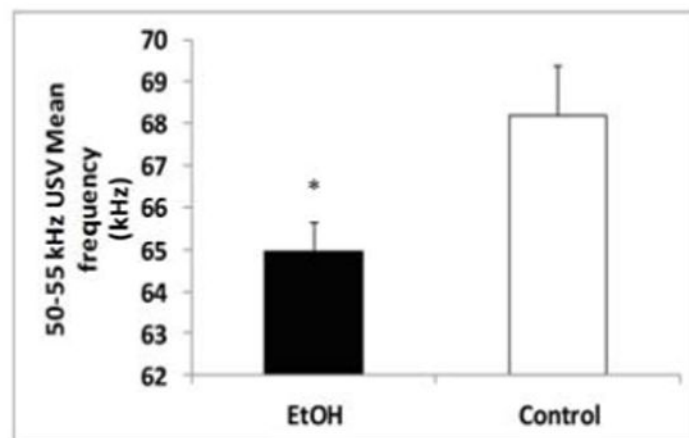
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(A)

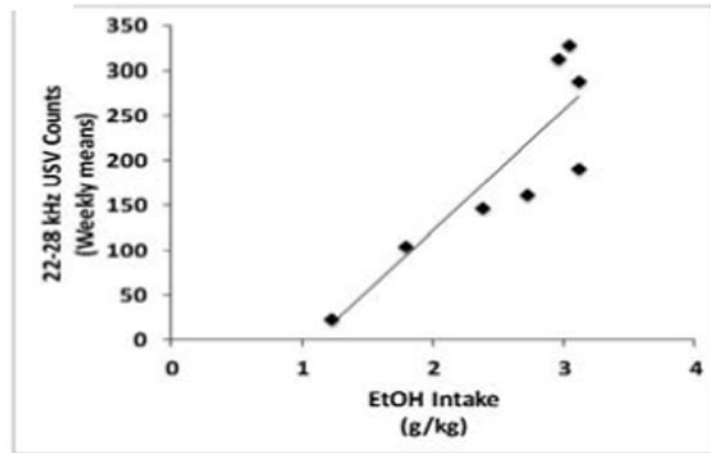


(B)

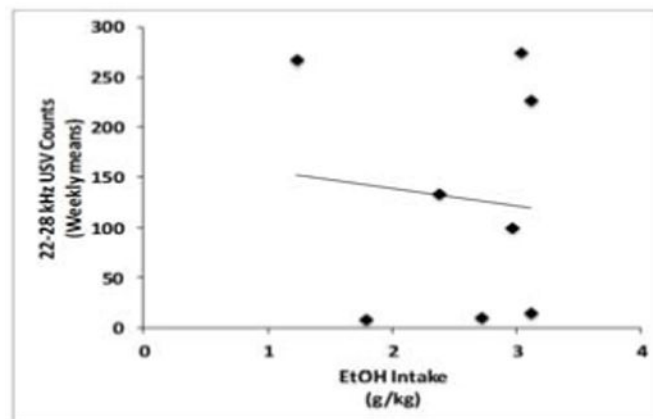


**Fig. 6.** USV mean frequency ( $\pm$  SEM) during EtOH ON intervals. **A.** A significant difference between EtOH ( $n=8$ ) and Control ( $n=6$ ) groups was detected in 22–28 kHz mean frequency ( $*=p<0.05$ ) when alcohol was available during DID sessions. **B.** The mean frequency of 50–55 kHz FM USVs was decreased in the EtOH group compared to the Control group during EtOH ON intervals ( $*=p<0.05$ ).

(A)

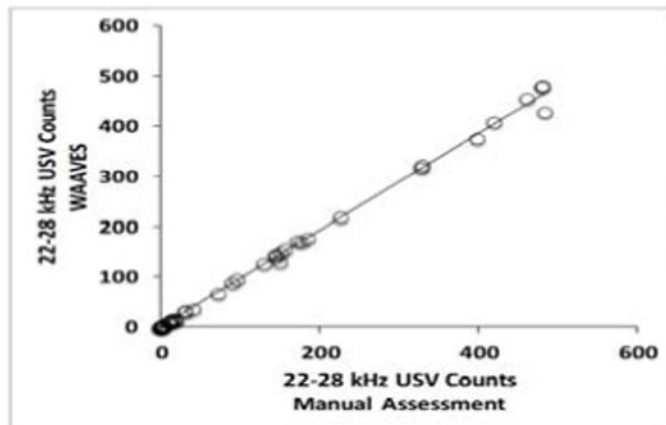


(B)

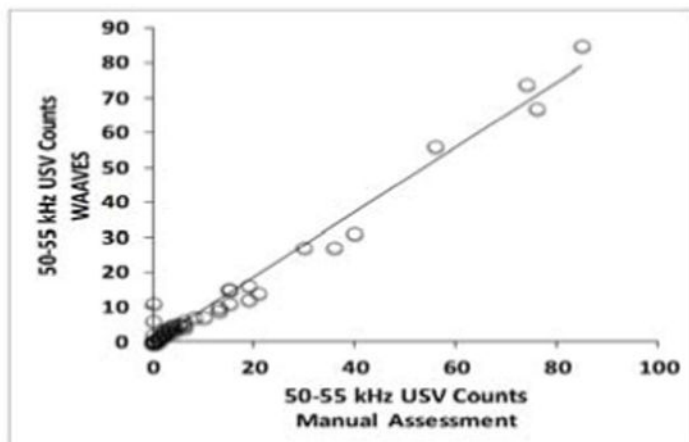


**Fig. 7.** Correlation between 22–28 kHz USVs ( $\pm$  SEM) emitted during EtOH OFF and ON intervals and EtOH Intake (g/kg). **A.** HAD-1 rats in the EtOH Condition ( $n=8$ ) displayed a significant correlation between EtOH intake and 22–28 kHz USVs during periods of EtOH OFF intervals ( $r = 0.87$ ;  $p < 0.01$ ). **B.** No significant linear relationship between EtOH Intake and 22–28 kHz USV counts was detected during EtOH ON intervals, ( $r = -0.11$ ;  $p = 0.8$ , n.s.).

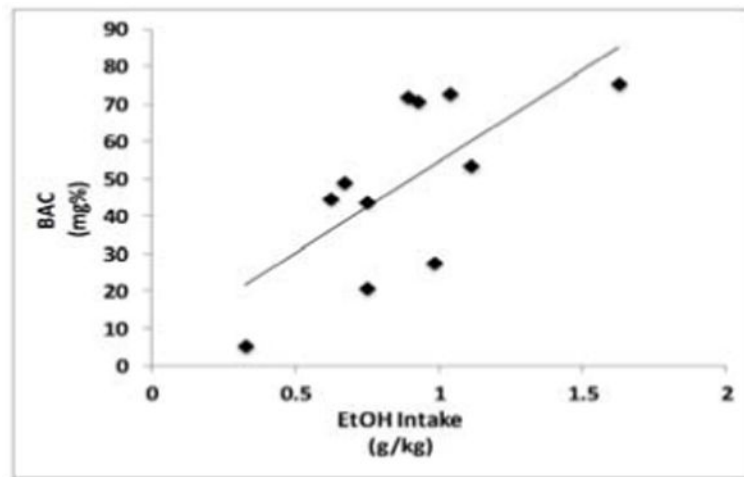
(A)



(B)

**Fig. 8.**

WAAVES ultrasonic vocalization (USV) count validation: correlation between WAAVES and human USV assessments. **A.** 22–28 kHz USV counts: from a sample of fifty 10-minute USV files, a highly significant linear relationship ( $r(50) = 0.99$ ;  $p < 0.001$ ) was revealed between USV counts assessed by WAAVES and manual (visual confirmation) techniques. **B.** 50–55 kHz frequency-modulated (FM) USV counts: fifty 1-minute USV files were used to verify WAAVES analyses by comparison with manual assessment (visual and auditory confirmation). A highly significant positive linear relationship exists between automated and manual USV assessments ( $r(50) = 0.99$ ;  $p < 0.001$ ).



**Fig. 9.** Alcohol intake validation: Correlation between blood alcohol concentration (mg%) and assessed EtOH dose. A significant positive linear relationship ( $r = 0.69$ ,  $p < 0.01$ ) between blood alcohol concentrations and assess EtOH intake confirmed measurement accuracy of intake in alcohol-experienced HAD-1 rats ( $n=11$ ). It is important to note that this dose range did not reach the average EtOH intake during DID sessions because of the abbreviated 30-minute interval during this test sessions compared to 3 hours of total EtOH access during DID sessions.