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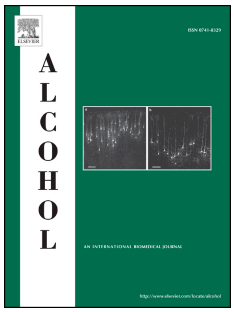


CUE-ALCOHOL associative learning in FEMALE RATS

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**ABSTRACT**

The ability of environmental cues to trigger alcohol seeking behaviors is believed to facilitate problematic alcohol use. We previously showed that the development of this cue-evoked alcohol approach reflects cue-alcohol learning and memory in the adult male rat; however, we do not know whether the same is true for similarly aged female rats. Consequently, adult Long-Evans female rats were allowed to drink unsweetened alcohol in the homecage (MWF 24 hr two-bottle choice, 5 weeks) and subsequently split into two experimental groups: paired and unpaired. Groups were matched for ingested doses and alcohol bottle preference across the pre-conditioning homecage period. Both groups were trained in conditioning chambers using a Pavlovian procedure. For the paired group, the chamber houselight was illuminated to signal access to an alcohol sipper. Houselight onset was yoked for the unpaired group, but access to the alcohol sipper was scheduled to occur only during the intervening periods (in the absence of light). We found that in the paired, but not unpaired group, an alcohol approach reaction was conditioned to houselight illumination, and the level of cue-conditioned reactivity predicted drinking behavior within trials. Groups experienced equivalently low but non-negligible blood alcohol concentrations over the course of conditioning sessions. We conclude that cue-triggered alcohol seeking behavior in adult female rats reflects associative learning about the relationship between alcohol availability and houselight illumination.

**Keywords:** oral alcohol; low dose; Pavlovian conditioning; female rat; cue reactivity

## INTRODUCTION

Environmental stimuli that have been routinely paired with alcohol can acquire the ability to trigger alcohol seeking behaviors and thereby contribute to problematic alcohol use. The implicit associative learning process that allows environmental stimuli paired with alcohol to acquire the ability to trigger alcohol seeking behaviors, Pavlovian or classical cue conditioning, is believed to operate in a fundamentally similar way in males and females. However, male and females may differ in level of susceptibility to specific ways in which cues can contribute to problematic alcohol use (Barker & Taylor, 2017). In the field of preclinical non-human animal models, there is a growing appreciation for qualitative and quantitative differences in the processes contributing to addiction-like behavior and its expression (e.g., drug cue learning, drug cue reactivity) between male and female individuals (Becker & Koob, 2016). In light of this growing appreciation of biological sex differences, the burden of proof is on researchers to demonstrate that our models of addiction-like behavioral phenomena in non-human animals operate similarly in males and females of the model organism species, and if not, to document the differences. Despite this, many preclinical studies of alcohol cue conditioning, especially those that use rats as the model organism and voluntary alcohol drinking paradigms, including our own (Cofresí et al., 2019; Cofresí, Lee, Monfils, Chaudhri, & Gonzales, 2018; Cofresí et al., 2017; Knight et al., 2016; Krank, 2003; Krank, O'Neill, Squarey, & Jacob, 2008; Lamb, Ginsburg, Greig, & Schindler, 2019; Lamb, Ginsburg, & Schindler, 2016; LeCocq, Lahlou, Chahine, Padillo, & Chaudhri, 2018; Millan, Reese, Grossman, Chaudhri, & Janak, 2015; Sparks, Sciascia, Ayorech, & Chaudhri, 2014; Srey, Maddux, & Chaudhri, 2015; Tomie, Festa, Sparta, & Pohorecky, 2003; Tomie, Kuo, Apor, Salomon, & Pohorecky, 2004; Tomie, Miller, Dranoff, & Pohorecky, 2006; Villaruel & Chaudhri, 2016), were conducted exclusively in male rats, and therefore, little is known about how Pavlovian alcohol cue conditioning proceeds in female rats.

Here, we determined if female rats were capable of associating an environmental stimulus with alcohol using the two-stage paradigm that we initially developed in male rats. In the first stage, we provide intermittent 24-hr access to unsweetened alcohol in the rat's homecage alongside free-access to food and water for 5 weeks. In the second stage, we test the ability of time-limited unsweetened alcohol drinking opportunities to condition alcohol seeking behavior to an antecedent visual cue in a physical environment different from the rat's homecage. In the latter test, a persistent change in the behavioral response to cue presentation could be due to learning to associate the cue with alcohol access or non-associative learning driven by repeated exposure to the cue or to alcohol. To distinguish between these possibilities, we characterized behavior during cue presentation in female rats that were trained on two versions of the same conditioning paradigm. In one, alcohol access was explicitly paired with houselight illumination (a visual cue), whereas in the other, the two events were explicitly *un*paired. Behavioral changes observed in the paired group, but not observed in the unpaired group reflect alcohol-associative learning about the visual cue. To examine whether persistent behavioral changes during visual cue presentation were driven by differences in the ability of rats in the paired and unpaired groups to consume alcohol during the visual cue conditioning sessions, we characterized consummatory behavior (sipper licking latency and intensity). To verify that rats in both groups had similar blood alcohol concentrations during visual cue conditioning sessions, we took blood samples at the end of a conditioning session, determined the relationship between ingested dose and blood alcohol concentration, and retrospectively predicted blood alcohol concentration at the end of each conditioning session as a function of ingested dose. In doing so, we also evaluated the extent to which changes in behavior across visual cue conditioning might be driven by alcohol's post-ingestive pharmacology. Finally, we tested whether conditioned behavioral reactivity to the visual cue for alcohol in the paired group predicted alcohol consummatory behavior and ingested dose, two predictions derived from

1 Tomie's model for how alcohol cue reactivity promotes problematic alcohol use (Tomie, 1996;
2 Tomie & Sharma, 2013).

3

## METHODS & MATERIALS

### Subjects

Subjects were adult female Long-Evans rats (Envigo; Indianapolis) weighing 200-225 g at arrival. Rats were singly housed in shoebox-style plexiglass homecages containing Sani-Chips® bedding and a Bio-Serv Gummy Bone (polyurethane; 5 cm L x 2.5 cm W). Metal wire cage-tops were used. Standard chow pellets were loaded into a large cup inside the cage. Tap water was provided via gravity-fed sipper inserted at approximately 45º from the cage top. Chow and water were replenished daily. Bedding was replaced weekly. Cages were located in a temperature and humidity controlled room (22±2 ºC). All procedures took place 4-5 hr into the light phase of a 12 hr light/dark cycle unless otherwise indicated. Drinking solutions were prepared from 95% ethyl alcohol (ACS/USP grade, Pharmco-AAPER) and tap water every 3 days. These were kept and served at room temperature (20 ºC). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin, and conducted in accordance with NIH guidelines.

### Pre-conditioning ethanol drinking in the homecage

This procedure was described in detail elsewhere (Cofresí et al., 2018, 2017; Sparks et al., 2014). Briefly, rats were provided a bottle of unsweetened ethanol (15% ethanol in tap water; v/v; 15E) and a new bottle of water for 24 hr every MWF for 5 weeks. Bottle placement on the cage-top alternated (ethanol on left- v. right-side) across sessions. Rats that failed to drink in week 1 were provided 5% and then 10% ethanol in tap water (v/v; 5E, 10E) to promote drinking. Any rats drinking < 1 g/kg/24 hr across week 5 were not retained for conditioning.

### Ethanol-reinforced classical conditioning

The conditioning chambers used were described in (Cofresí et al., 2017). The conditioning procedures were previously described (Cofresí et al., 2019). Briefly, rats were assigned to "Paired" or "Unpaired" conditioning such that the resulting groups were matched for ingested doses across the 5 weeks of pre-conditioning drinking sessions. Rats in both groups were trained to use the retractable ethanol sipper in the conditioning chamber and habituated to chamber houselight illumination. Rats then underwent cue conditioning across 12 consecutive days. Each conditioning session consisted of 8 trials. The inter-trial interval (ITI) was variable (mean: 280 s, min: 160 s, max: 360 s). After a 5 min wait period, the session started (with the first ITI). This was signaled to the rat by onset of the exhaust fan. The session ended when the final ITI (selected after trial 8) elapsed. This was signaled by offset of the exhaust fan inside each cubicle. During each trial in a session, the chamber houselight was illuminated for 20 s. In the Unpaired group, there was no consequent event. In the Paired group, the retractable bottle assembly was activated 10 s into the illumination to present a metal sipper such that ethanol access and houselight illumination co-terminated. Sipper presentations for the Unpaired group occurred mid-ITI, beginning in ITI 2 and ending in ITI 9. Houselight illumination onset, offset, and ITIs were yoked between groups. Sipper presentations were yoked within groups. Licking the sipper produced 10E or 15E, whichever the rat was drinking at the end of the pre-conditioning phase.

**Blood collection & ethanol analysis**

After the 12$^{th}$ conditioning session, 1-2 additional sessions were given. At the end of one of these sessions, blood was sampled from the lateral saphenous vein while the rat was under isoflurane anesthesia. Ethanol concentration (mg/dL) in the blood sample was determined using gas chromatography with flame ionization detection as in (Carrillo et al., 2008; Cofresí et al., 2019, 2018).

**Behavior Measurement**

Trials from conditioning sessions 1-12 were sampled for behavior from digital video recordings. As in (Cofresí et al., 2019, 2018, 2017; Lee et al., 2005), instantaneous observations were made every 1.25 s starting 5 s before houselight illumination such that there were 4 observations per 5 s bin across the illumination period. At each observation, the rater noted the absence or presence of mutually-exclusive behavioral states (sipper site approach: approaching or exploring the sipper insertion hole; orienting to light: both forepaws off the floor, supported by hindlimbs; other: grooming, resting). Each of 5 s bins corresponded to a meaningful trial phase. Since the original paradigm (group Paired) was designed with houselight illumination as the conditional stimulus (CS), the bins are labeled with reference to the CS. The preCS bin is the 5 s period before CS presentation and CS bins 1-4 are the 5 s periods across CS presentation. Sipper licking was automatically recorded using a contact lickometer circuit. The latency (s) to first lick was also recorded on every trial. If no lick was registered within 10 s of sipper onset, then a maximum latency of 10 s was recorded. A second, modified contact lickometer circuit was used to automatically record forepaw contacts with the area of the chamber wall immediately around the sipper insertion hole independently of sipper licking, as described in (Cofresí et al., 2019). The latency (s) to first forepaw contact after houselight onset was recorded. If no forepaw contact was registered within 30 s of houselight onset, then a maximum latency of 30 s was recorded. The latency (s) to first forepaw contact after sipper onset was also recorded. If no forepaw contact was registered within 20 s of sipper onset, then a maximum latency of 20 s was recorded. For both post-houselight and post-sipper onset forepaw contact latencies, if sustained forepaw contact was on-going at the time of houselight/sipper onset (viz., if the rat was “holding on” to the area around the sipper insertion hole), then a negative latency was recorded because initiation of on-going contact was at an earlier time than onset of the

houselight/sipper. Infrared photo-beams were used to track general locomotion in the stimulus rich (houselight fixture and sipper hole) v. poor (bare wall) zones of the conditioning chamber.

The dose of ethanol ingested by each rat was also monitored. For every homecage drinking session, bottles on an empty control cage were used to measure loss due to evaporation and spillage and correct drinking solution intake values across all subjects. For every conditioning session, a weigh boat underneath each bottle assembly collected spillage and drinking solution intake values were corrected at the level of each individual subject. Drinking solution intake was measured as the corrected mass difference in bottle weight pre- and post-session. To obtain the ingested ethanol dose, the amount (g) of pure ethanol consumed was computed and expressed relative to body weight (kg) each rat.

**Statistical Analysis**

Mixed factorial analysis of variance (ANOVA) was the primary statistical analysis technique used to analyze behavioral and drinking data. The threshold for statistical significance was $p<0.05$. Significant results in the omnibus ANOVA were followed up as appropriate (e.g., ANOVA F-tests were used to decompose interactions of 2 or more factors, t-tests were used to decompose the main effect of a factor). Bonferroni correction was applied at every follow-up stage to minimize false discovery. In few instances, we used other statistical procedures. However, the threshold for statistical significance remained $p<0.05$ for these other analyses. For example, we used Pearson's correlation test to evaluate the relationship between blood ethanol and ingested dose.

All analysis was done in R version 3.5.1 (R Core Team, 2018) using the car package (Fox & Weisberg, 2011). Data were plotted in R using the ggplot2 package (Wickham, 2009) and finalized in Inkscape version 0.92.2 (Inkscape Team, 2017).

## RESULTS

Of 20 rats obtained for the study, 19 were conditioned on the basis of *a priori* retention criterion: ingested dose ≥ 1 g/kg/24hr on average across the last week of the pre-conditioning phase. Pre-conditioning homecage ethanol drinking data are presented in **Supplemental Figure 1**.

Of the 19 rats that were conditioned, 17 were retained based on our *a priori* inclusion criterion: ingested dose ≥ 0.30 g/kg/session on average across the last 3 conditioning sessions. The 2 rats that failed to meet the latter criterion were both in group Unpaired. One of those 2 had been conditioned with 10E, and the other had been conditioned with 15E. Of the 17 that met our *a priori* inclusion criterion, all 7 in group Unpaired and 8 out 10 in group Paired had been conditioned with 15E. The remaining 2 out of 10 rats in group Paired that met our *a priori* inclusion criterion had been conditioned with 10E.

During the waiting period before the first conditioning session, rats in group Paired and Unpaired alike were more active in the stimulus-rich half of the conditioning chamber (i.e., with the houselight fixture and sipper insertion hole) than the stimulus-poor half, and this did not change over the course of conditioning (**Supplemental Figure 2A**). Rats in group Paired and Unpaired alike, however, did make increasingly more forepaw contacts with the area around the sipper insertion hole during the pre-session waiting period over the course of conditioning (**Supplemental Figure 2B**).

<u>Drinking in the conditioning chamber across ethanol-reinforced classical conditioning</u>

Rats in group Paired and Unpaired drank similarly across the conditioning phase. Ingested doses increased significantly across conditioning sessions (session main effect: $F_{11,165}$=26.08, p<0.001), and the pattern of increase was similar between groups (group main effect and group x session interaction: NS; **Figure 1A**).

Seventeen rats met our *a priori* minimum drinking criteria across conditioning session 10-12. We wanted to monitor the blood ethanol concentrations achieved after the conditioning sessions, but we wanted to avoid the possible effects of the invasive blood sampling procedure on the behavior in subsequent sessions. Therefore, the rats were exposed to 1 or 2 additional conditioning sessions, and blood was sampled 8-11 min after the 8$^{th}$ sipper presentation. Blood ethanol concentration (BEC) at the end of the conditioning session was significantly related to ingested dose (Pearson's $r$ = +0.76, $t_{15}$=4.60, p<0.001; **Figure 1B**). Body weights ranged from 260 to 318 g. Ingested dose ranged from 0.35 to 1.2 g/kg with a mean ± sem of 0.72 ± 0.05 g/kg. BEC ranged from 0 to 38.5 mg/dL with a mean ± sem of 15.9 ± 3.4 mg/dL. Groups Paired and Unpaired did not differ in BEC, ingested dose, the relationship between dose and BEC, sampling time, or body weight on blood sampling day (**Table 1**). Thus, a single simple regression equation was used to predict BEC as a function of ingested dose across the conditioning sessions. Estimated end of session BEC across conditioning sessions did not differ between group Paired and Unpaired (session main effect: $F_{11,165}$=10.67, p<0.001; group main effect and group x session interaction: NS; **Figure 1C**). Overall, end of session estimated BEC were low but non-zero after session 6.

Acquisition of houselight cue-triggered ethanol seeking in group Paired, but not Unpaired

Groups Paired and Unpaired differed in sipper site approach frequency during the trial phases across training (group x session interaction: $F_{11,\ 165}$=5.94, p<0.001; group x trial phase interaction: $F_{2,\ 30}$=5.18, p<0.05; **Figure 2A**). For rats in the Paired group, sipper site approach frequency increased over sessions (simple session effect: $F_{11,\ 99}$=12.03, p<0.001) and as a function of houselight illumination period (simple trial phase effect: $F_{2,\ 18}$=4.78, p<0.05; pairwise t-tests for preCS bin < CS bin 1 and CS bin 1 < CS bin 2: $t_9$>5.25, p<0.0001). In contrast, sipper site approach frequency remained at floor across trial phases and sessions for rats in group Unpaired (simple session & trial phase effects: NS). The difference in sipper site approach level

was clearest in CS bin 2 (simple group effect: $F_{1, 15}$=19.78, p<0.001; **Figure 2A** rightmost panel).

To confirm these findings, we also analyzed sipper site (faceplate) contact frequency, which was measured automatically using a modified lickometer circuit, and thus, free of rater bias. Results were similar to those presented above. Groups Paired and Unpaired differed in sipper site contact frequency across training (group main effect: $F_{1, 15}$=5.78, p<0.03; session main effect: $F_{11, 166}$=4.18, p<0.001; group x session interaction: $F_{11,165}$=5.16, p<0.001), but the group x trial phase interaction effect was not statistically significant ($F_{2, 30}$=1.81, NS). However, it can be seen in **Figure 2B** that for rats in the Paired group, sipper site contact frequency during CS bin 1 and 2 increased over sessions whereas contact during the preCS bin remained at floor. In contrast, sipper site contact frequency remained at floor across sessions in every bin for rats in group Unpaired (**Figure 2B**).

The frequency of houselight illumination-elicited orienting across sessions is presented in **Supplemental Figure 3**.

Houselight cue-elicited ethanol seeking reaction dynamics in session 12

Our previous studies in group Paired male rats found that the ability of the houselight cue to elicit ethanol seeking appears to decrease across trials within sessions reliably by conditioning session 12 (Cofresí et al., 2019, 2018). In order to verify whether the same behavior pattern occurs in group Paired female rats, we examined trial by trial behavior in conditioning session 12. Overall, female rats in group Paired exhibited a robust sipper site approach reaction to houselight illumination in session 12 whereas those in group Unpaired did not (group main effect: $F_{1, 15}$=13.93, p<0.003; **Figure 3A**). Focusing on trial phase CS2, sipper site approach frequency was greater for group Paired than Unpaired in every trial ($t_{15}$≥2.10, p≤0.05) (**Figure 3A**).

Similar results were obtained when we analyzed per-trial sipper site contacts (i.e., forepaw contact with the faceplate around the sipper insertion hole) in session 12. Overall, rats in group Paired made many contacts after houselight onset whereas those in group Unpaired made few to no contacts (group main effect: $F_{1, 15}$=6.23, p<0.025; **Figure 3B**).

The per-trial frequency of houselight illumination-elicited orienting in session 12 is presented in **Supplemental Figure 4**.

Acquisition of similar reactions to sipper presentation across ethanol-reinforced classical conditioning in group Paired and Unpaired

Equipment malfunction resulted in failure to record sipper licking during at least one session for 1 rat in group Unpaired, reducing sample size to 6 for these analyses.

There was a decrease across sessions in average latency to start licking per trial (session main effect: $F_{11, 154}$=12.58, p<0.001; **Figure 4A**). There was a concomitant increase across sessions in average licks per trial (session main effect: $F_{11, 154}$=16.57, p<0.001; **Figure 4B**). Statistically significant group x session interaction effects were also detected (in latency: $F_{11,154}$=2.05, p<0.05; in licks: $F_{11,154}$=3.15, p<0.05), but simple effects decomposition revealed that these were driven by trivial differences between groups early in conditioning (sessions 1, 2 and/or 3) that were not statistically significant after Bonferroni correction (**Figure 4A-B**). Importantly, by the end of conditioning, there was no significant difference between group Paired and Unpaired in either the average latency to start licking or the average licks per trial (both group main effects, both session main effects, and both group x session interactions over sessions 10-12: NS before and after Bonferroni correction; **Figure 4A-B**).

Correlation between cue-elicited ethanol seeking and ethanol drinking behavior in group Paired

For ease of comparison to (Cofresí et al., 2018), each group Paired rat's asymptotic level of behavior was estimated as the average across conditioning session 10-12. The cue-elicited ethanol seeking reaction was indexed by the level of approach during trial phase CS2 per trial because that is the within-trial period during which it was at its peak. Indices of ethanol drinking behavior included latency to start licking the sipper per trial, the number of licks per trial, and the total ingested dose of ethanol per session.

Cue-elicited ethanol seeking explained 61% of the total variance in the average latency to lick the sipper, 58% of the variance in the average numbers of licks, and 40% of the variance in the average total ingested dose in group Paired. Specifically, we found that on average across trials at asymptote, higher levels of cue-elicited ethanol seeking were significantly associated with lower latency to start ethanol sipper licking ($r$ =-0.78, t(8)=-3.53, p<0.01; **Figure 5A**), more licking ($r$ =+0.76, t(8)=+3.15, p<0.02; **Figure 5B**), and larger ingested doses ($r$ =+0.63, t(8)=+2.314, p<0.05; **Figure 5C**).

## DISCUSSION

In the present study, we had the following goals: (1) determine whether cue-triggered alcohol-seeking behaviors in female rats resulted from repeated exposure to alcohol, the cue, or associative learning; and (2) test whether the covariation between alcohol cue reactivity and drinking behavior existed within-episodes as predicted by a major theoretical framework for understanding the role of Pavlovian alcohol cues in alcohol use behavior.

### Pre-conditioning free-choice alcohol drinking & preference

Adult female rats drank just as much alcohol at the start as at the end of the 5-week pre-conditioning homecage drinking period in the present study (**Supplemental Figure 1A**) which replicates previous findings (Butler, Carter, & Weiner, 2014; Morales, McGinnis, & Mccool, 2015). Unlike in those studies, however, our female rats appeared to lose their initial aversion to

the taste of unsweetened alcohol (**Supplemental Figure 1B**). This could be accounted for by the rats learning to associate the taste of alcohol with its post-ingestive reinforcing effects (metabolic or pharmacological or both). This minor discrepancy between our study and the studies of Morales et al. (2015) and Butler et al. (2014) is most likely attributable to our use of a lower alcohol concentration in the drinking solution (10-15% alcohol v/v in tap water in our study compared to 20% alcohol v/v in their studies).

Acquisition of alcohol cue reactivity

After acquisition of voluntary drinking, we tested for cue-alcohol associative learning. The only difference between the two groups (paired and unpaired) was the presence of a positive contingency between houselight illumination (CS) and alcohol access in the paired group. In both groups, rats learned to react to sipper presentation with rapid initiation of vigorous consummatory licking (**Figure 4A-B**), and learned to react to initial oral alcohol receipt with an increase in the rate of consummatory licking (**Supplemental Figure 6A**)**.** Rats in both groups ingested similar doses of alcohol across conditioning (**Figure 1A**). Similar levels of alcohol were detected in blood approximately 10 min after the 8$^{th}$ drinking opportunity in a conditioning session (**Figure 1B**), and similar levels were predicted to be experienced over the course of conditioning (**Figure 1C**). Although total ingested doses by these female rats were numerically larger than those ingested by male rats in the same paradigm, blood alcohol levels in the female rats were similar to those of male rats in this (Cofresí et al., 2019, 2018) and similar paradigms (LeCocq et al., 2018). During the 5-min pre-session “waiting” periods, both groups moved around more in the stimulus-rich than stimulus-poor side of the conditioning chamber (**Supplemental Figure 2A**) and made a similar number of sipper site (faceplate) contacts (**Supplemental Figure 2B**). However, only rats in paired group acquired houselight illumination-elicited anticipatory sipper site approach and contact behavior (**Figure 2A-B**). This is strong behavioral evidence that cue-triggered alcohol seeking behaviors arise from associative

learning and not merely repeated exposure to alcohol or the cue within the same context. Additionally, it confirms that associative learning about antecedent conditional stimuli for alcohol access in this (Cofresí et al., 2019) and similar paradigms (Srey et al., 2015) is not restricted to male rats.

Despite equivalent alcohol exposure, rats in the unpaired group did not develop cue-triggered alcohol-directed behavior. However, we did observe persistence of the overt attentional orienting reaction to houselight illumination—specifically, orienting during the second half of light illumination (**Supplemental Figure 3-4**)—in the unpaired female rats. We have also observed the same form of persistent orienting in male rats which went through a similar habituation and conditioning paradigm with the houselight being explicitly unpaired with alcohol access (Cofresí et al., 2019). Our present findings in female rats suggest that in both sexes, the persistent overt attentional response in the unpaired groups may be a conditioned attentional response (Holland, 1980; Delamater & Holland, 2008) that reflects associative learning about houselight *offset* as a predictor of alcohol access.

Trial-by-trial dynamics of alcohol cue reactivity

In the present study, in conditioning session 12, the paired group female rats exhibited no within-session trial-by-trial decay in the level of houselight illumination-elicited sipper site approach and contact (**Figure 3A-B**). Female rats in the paired and unpaired groups alike exhibited no trial-by-trial change in the latency to approach the sipper area upon sipper presentation (**Supplemental Figure 5A**), but did exhibit a small trial-by-trial increase in the latency to start drinking (**Supplemental Figure 5B**), and a small decrease in the overall intensity of drinking from trials 1-4 to 5-8 (**Supplemental Figure 6B**). In contrast, in our previous study, equivalently experienced, paired group male rats exhibited trial-by-trial decreases in the

vigor of both houselight illumination-elicited sipper site approach and drinking behavior whereas male rats in unpaired group did not (Cofresí et al., 2019, 2018).

We explained our previous findings by positing that rats may experience progressive within-session specific satiety for alcohol, and consequently, progressive devaluation of the alcohol reinforcer (Samson, Czachowski, & Slawecki, 2000; Samson, Slawecki, Sharpe, & Chappell, 1998). Cue-elicited goal-directed behavior is known to be sensitive to between-session reinforcer devaluation (e.g., specific satiety, pairing with illness) in food and sugar cue conditioning paradigms (Holland & Rescorla, 1975; Morrison, Bamkole, & Nicola, 2015). On the basis of that literature, we argued that if within-session specific satiety for alcohol and consequent devaluation of the alcohol reinforcer were taking place, then we would expect trial-by-trial decay in the level of houselight cue-elicited alcohol seeking. The present findings suggest that while cue-elicited alcohol seeking may be sensitive to progressive within-session specific satiety for alcohol and consequent devaluation of the alcohol reinforcer in male rats, it may not be similarly sensitive in female rats.

We also previously argued that if the *vigor* of alcohol drinking behavior had come to be in part controlled by the conditioned alcohol cue, then it too would be sensitive to progressive within-session satiety for alcohol and consequent devaluation of the alcohol reinforcer. If so, then we would expect a trial-by-trial decrease in the *vigor* of alcohol drinking behavior specifically among the paired, but not unpaired, group female rats. Given that both paired and unpaired groups in the present study exhibited trial-by-trial decreases in the vigor of alcohol drinking behavior (**Supplemental Figure 5B** and **6B**), we cannot argue that the conditioned alcohol cue in paired group female rats exerted any direct control over the vigor of their alcohol drinking behavior. However, our finding that both paired and unpaired group female rats exhibited trial-by-trial

decreases in alcohol drinking behavior agrees with the idea that progressive within-session specific satiety took place.

Thus, it is tempting to interpret the insensitivity of cue-elicited alcohol seeking to within-session specific satiety for alcohol in the female rat as an indication that despite conditioning the alcohol cue at a similar rate and reacting to that cue with what looks like the same response, male rats encoded the alcohol cue in a stimulus-outcome memory whereas female rats encoded the alcohol cue in a stimulus-response memory. A more parsimonious, and more easily tested, alternative explanation is that male and female rats may simply be differentially sensitive to different types of reinforcer devaluation in general or specifically, different types of devaluation applied to an alcohol reinforcer. Either explanation has implications for the sensitivity of alcohol cue reactivity to different behavioral interventions between men and women.

Cue-triggered alcohol-directed reactivity promotes alcohol intake

According to a model for alcohol abuse proposed by Tomie and colleagues (Tomie, 1996; Tomie & Sharma, 2013), alcohol cue reactivity should co-vary with alcohol drinking. One of our previous studies confirmed this prediction in male rats (Cofresí et al., 2018). In the present study, we extend this finding to female rats. Specifically, we found that greater levels of houselight illumination-elicited alcohol seeking predicted faster initiation of drinking, more drinking, and the ingestion of larger alcohol doses (**Figure 5A-C**). These relationships could be due to a causal response chain or between-subject differences in biopsychological factors that influence conditioning rates, reactivity levels, and drinking.

The present study in context

Our present findings are not surprising given that female rats have been shown to condition behavioral reactions to cues predicting: (1) appetitive stimuli such as food or sugar pellets

(Anderson & Petrovich, 2015; Pitchers et al., 2015); (2) aversive stimuli such as mild foot shock (Kosten, Lee, & Kim, 2006; Milad, Igoe, Lebron-Milad, & Novales, 2009; Pryce, Lehmann, & Feldon, 1999); and (3) drugs of abuse such as cocaine (Feltenstein, Henderson, & See, 2011; Kippin et al., 2005), especially in involuntary drug exposure paradigms (Bobzean, Dennis, & Perrotti, 2014; Campbell, Wood, & Spear, 2000; S. J. Russo et al., 2003; Scott J. Russo et al., 2003). In fact, there is evidence for appetitive and aversive conditioning to cues predicting involuntary alcohol exposure in female rats (Nentwig, Myers, & Grisel, 2017; Sherrill, Berthold, Koss, Juraska, & Gulley, 2011; Torres, Walker, Beas, & O'Dell, 2014). Additionally, there is indirect evidence for female rats conditioning to appetitive cues for voluntary alcohol consumption from studies of cue-induced reinstatement of extinguished alcohol self-administration behaviors (Bertholomey, Nagarajan, & Torregrossa, 2016; Randall, Stewart, & Besheer, 2017). The main contribution of the present study to our field is as an empirical demonstration that appetitive Pavlovian conditioning to voluntary alcohol consumption progresses similarly in female as well as male rats. Our unequivocal verification of this basic phenomenon in female rats is important for other pre-clinical laboratories conducting behavioral or neurobiological studies of alcohol cue reactivity using the rat as a model organism.

On the role of the estrous cycle

The present study was not designed to evaluate estrous cycle effects on alcohol learning & memory in the freely cycling female rat. In fact, we chose not to monitor the estrous cycle in our study for two reasons. First and foremost, we wanted to minimize procedural differences between the present study and our previous studies in male rats. Second, we were concerned that daily vaginal lavage could be capable of altering the conditioning properties of alcohol because it is an invasive, stressful procedure (Sharp, Zammit, Azar, & Lawson, 2003). Others have applied daily lavage and not observed detrimental effects on homecage alcohol drinking

and operant self-administration (Priddy et al., 2017).[1] Importantly, (Priddy et al., 2017) also reported null effects of estrous cycle phase in agreement with earlier studies in freely cycling female rats (Roberts, Smith, Weiss, Rivier, & Koob, 1998). Another recent study, in which vaginal lavage was done only once after the final operant self-administration session, also failed to find an effect of estrous cycle phase in freely cycling female rats (Bertholomey et al., 2016). Despite these null effects of the estrous cycle on alcohol intake in female rats, a recent meta-analysis across human and non-human animal models indicated that gonadal hormones do exert modulatory effects on alcohol intake (Erol, Ho, Winham, & Karpyak, 2017). Moreover, failure of the estrous cycle to modulate overall voluntary alcohol consumption does not preclude the estrous cycle from modulating cue reactivity phenomena. Indeed, extinction of fear and cocaine cues, and especially, post-extinction relapse-like return of reactivity to those cues, have been shown to be modulated by estrous cycle phase in the female rat (Feltenstein et al., 2011; Kippin et al., 2005; Milad et al., 2009). Additionally, studies of conditioned place preference to involuntary alcohol exposure in female rats (Torres et al., 2014) and female mice (Hilderbrand & Lasek, 2018) alike strongly implicate gonadal hormone variation over the estrous cycle in modulating the appetitive conditioning properties of alcohol. Consequently, future studies should characterize the role of the female rat estrous cycle, if any, in appetitive Pavlovian conditioning to voluntary alcohol consumption, its extinction, and post-extinction relapse-like response return.

Conclusion

We found that an alcohol access-related cue acquired the ability to elicit an alcohol approach response in female rats only if that cue positively predicted alcohol access. In doing so, we confirmed that associative learning about antecedent conditional stimuli for alcohol access in

[1] However, it should be noted that, to our knowledge, no proper experiment evaluating the potential effects of daily vaginal lavage (as a stressor) on the alcohol intake of female rats could be found in the literature.

our paradigm, by extension in similar paradigms, is not restricted to male rats. Within-session patterns of cue-elicited alcohol seeking and drinking by female rats exhibited subtle differences from what we have previously observed in male rats. Overall, our findings underscore the importance of Pavlovian conditioning processes in alcohol self-administration across the sexes as well as the need for increased study of the female sex in preclinical animal models of alcohol cue reactivity.

**ACKNOWLEDGEMENTS**

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## FIGURE CAPTIONS

**Figure 1. Equivalent ethanol exposure across cue conditioning. A:** Ingested dose (g/kg) per session shown across conditioning sessions in all the animals. Horizontal line shows *a priori* inclusion criterion (dose ≥0.30 g/kg/session across sessions 10-12). **B:** Relationship between blood ethanol concentrations detected approximately 10 min after the 8$^{th}$ 10-s drinking opportunity in a conditioning session and total ingested ethanol doses in the same session for adult, female Long-Evans rats. Black and white triangles represent group Paired (n=10) and Unpaired (n=7), respectively. Regression line and 95% confidence limits shown by solid line and shaded area, respectively. **C**: Mean ± sem estimated blood ethanol concentrations across conditioning sessions (approximately 10 min after the 8$^{th}$ drinking opportunity in the session) using ingested doses from **A** and regression equation from **B** for the same 17 rats.

**Figure 2. Conditioning of houselight-elicited anticipatory seeking.** Mean±sem level of sipper site approach (**A**) and faceplate contacts (**B**) paneled by trial phase (preCS bin: 5 s bin before houselight onset; CS bins 1-2: 1$^{st}$ and 2$^{nd}$ 5 s bin of illumination) shown across conditioning sessions (8 trials/session, 1 session/day, 12 consecutive days) for adult, female Long-Evans rats. Black and white triangles represent group Paired (n=10) and Unpaired (n=7), respectively. Approach data (maximum response level was 4) were derived from offline manual videoscoring (see main text Methods: Behavior Measurement for videoscoring details). Contact data were collected online using a modified lickometer (see main text Methods: Behavior Measurement for details).

**Figure 3. Within-session dynamics of houselight-elicited anticipatory seeking.** Mean ± sem level of anticipatory sipper site approach (**A**) and faceplate contacts (**B**) in the 5 s before light onset (bin -1) and over the 10 s post-light onset but pre-sipper onset (CS bin 1 and 2, each 5 s) paneled by trial (1-8) within conditioning session 12 for adult, female Long-Evans rats.

Black and white triangles represent group Paired (n=10) and Unpaired (n=7), respectively. Approach data (maximum response level was 4) were derived from offline manual videoscoring.

**Figure 4. Equivalent drinking behavior across cue conditioning.** Mean±sem (**A**) latency (s) to start licking per trial and (**B**) number of licks per trial shown across conditioning sessions (8 trials/session, 1 session/day, 12 consecutive days) for adult, female Long-Evans rats. Black and white triangles represent group Paired (n=10) and Unpaired (n=6 out of 7 due to equipment malfunction), respectively.

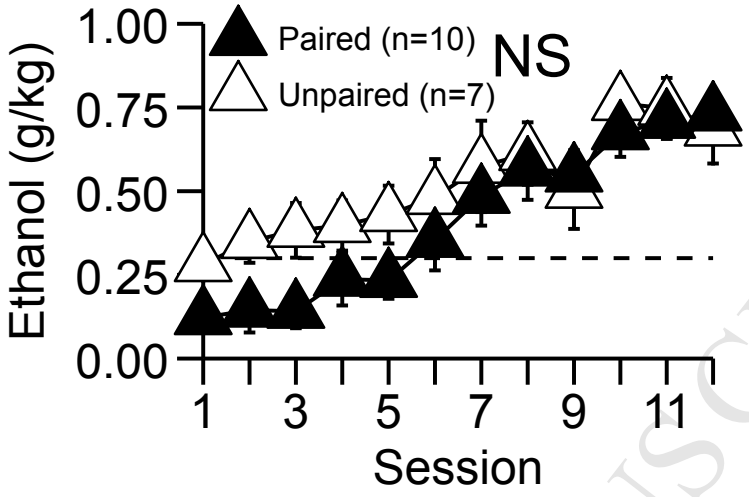
**Figure 5. Conditioned cue reactivity predicts drinking latency, drinking intensity, and ingested dose in group Paired.** Relationships of latency to start licking per trial (**A**), total licks per trial (**B**), and ingested dose per session (**C**) to houselight-elicited sipper site approach level per trial (maximum = 4) on average across conditioning sessions 10-12. Data were from 10 adult, female Long-Evans rats. Solid lines in each panel represent the regression line. Dashed lines represent the upper and lower 95% confidence limits around the regression line.

**Table 1. Blood ethanol concentrations, bodyweight, and drinking between groups on blood sampling day**

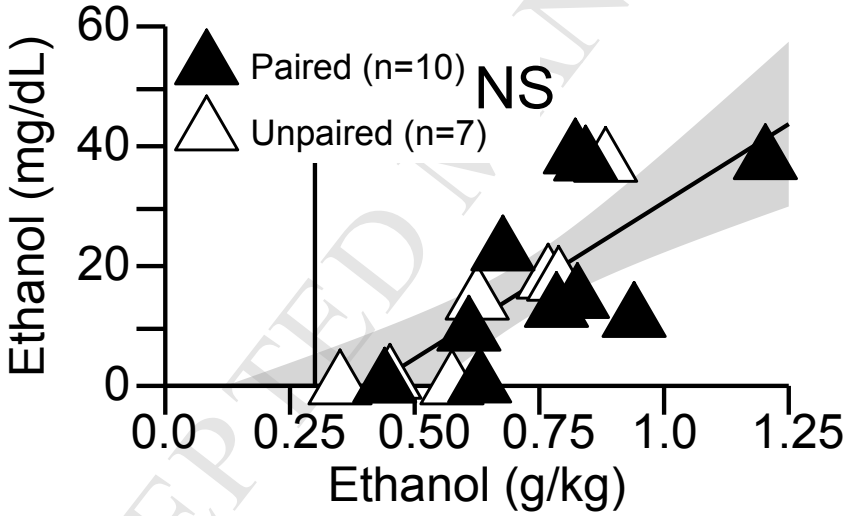
| | Paired (n=10) | Unpaired (n=7) | |
|---|---|---|---|
| BEC (mg/dL) | 18.41±4.70 | 12.46±5.13 | $T_{15}$=0.839, NS |
| Time after 8th sipper presentation (min) | 9.09±0.25 | 9.33±0.33 | $T_{15}$=-0.588, NS |
| Bodyweight (g) | 283.3±5.23 | 282.0±3.72 | $T_{15}$=0.185, NS |
| Ethanol (g) | 0.219±0.017 | 0.178±0.020 | $T_{15}$=1.529, NS |
| Dose (g/kg) | 0.777±0.0658 | 0.634±0.072 | $T_{15}$=1.438, NS |
| *BEC-Dose Correlation Coefficient* | 0.677 (0.08, 0.92)* | 0.892 (0.42, 0.98)* | $T_{13}$=0.552, NS |

BEC stands for blood ethanol concentration. For rows 2-6, entries in columns 2-3 are M ± SEM. For row 7, entries in columns 2-3 are Pearson's product-moment correlation coefficients with lower and upper 95% confidence limits in parentheses. Asterisks indicate $p<0.05$ for the null hypothesis that the true correlation coefficient equals zero. For rows 2-6, entries in column 4 are Student's t-test results for the null hypothesis that the true mean difference between groups equals zero. For row 7, the entry in column 4 represents the Student's t-test result for the null hypothesis that group does not moderate the relationship between BEC and dose.

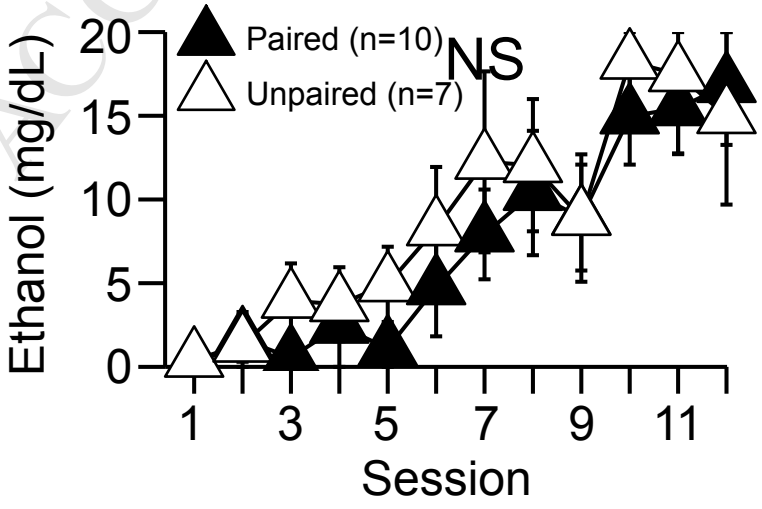
## A. Ingested Doses Across Conditioning

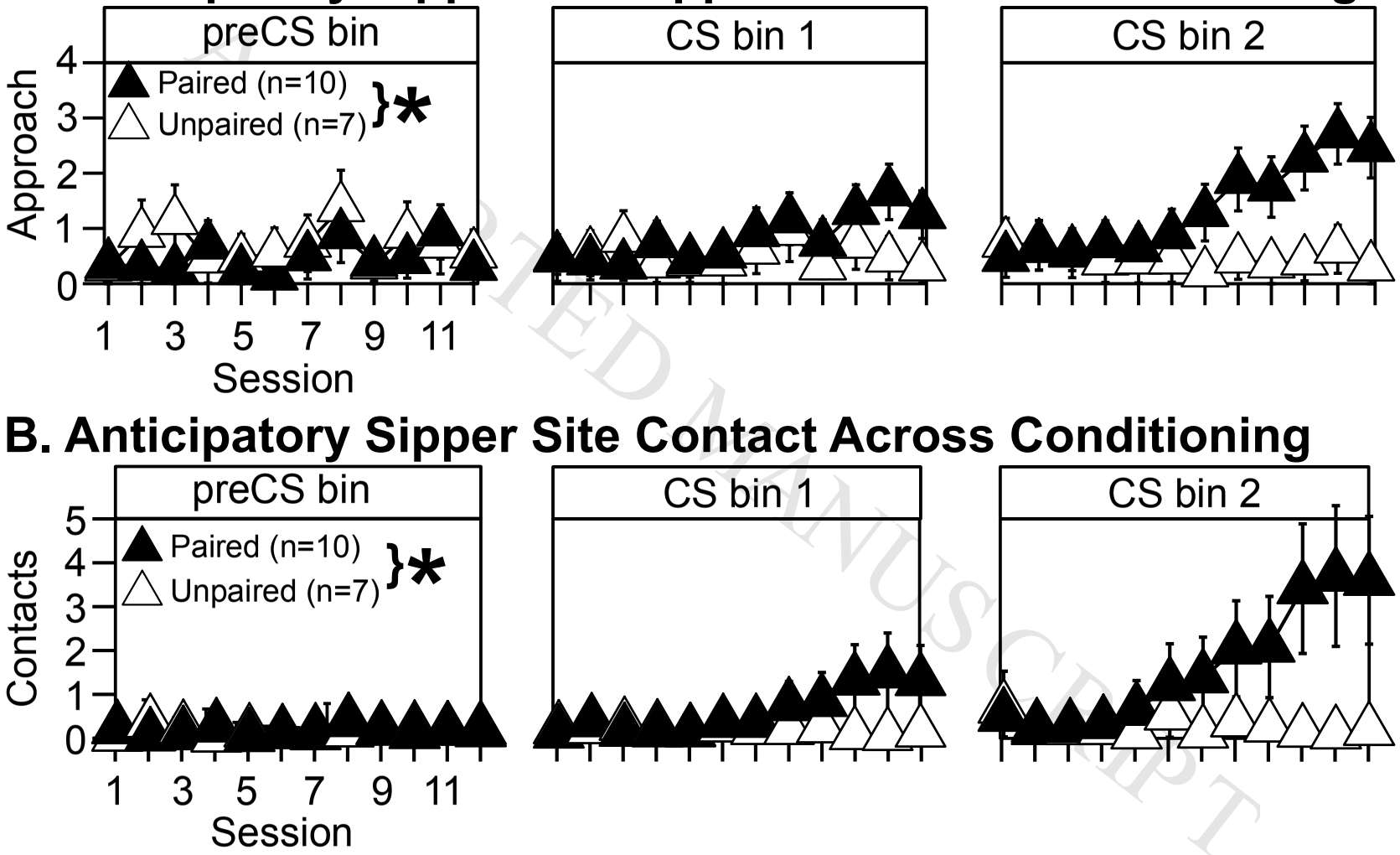



## B. Measured Post-Session Blood Ethanol

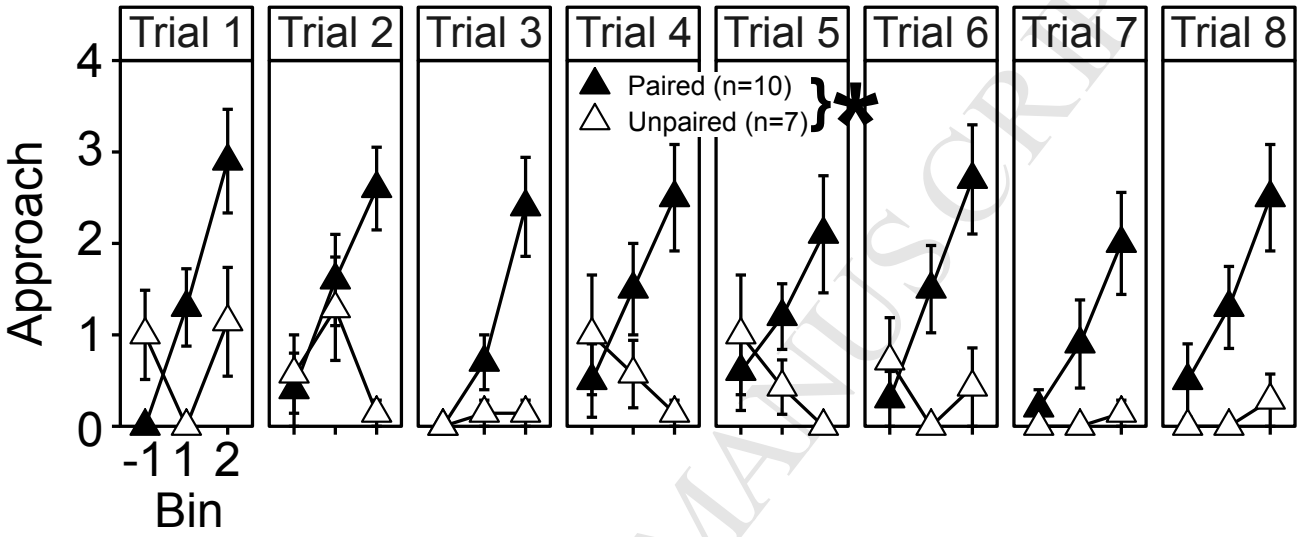



## C. Estimated Post-Session Blood Ethanol

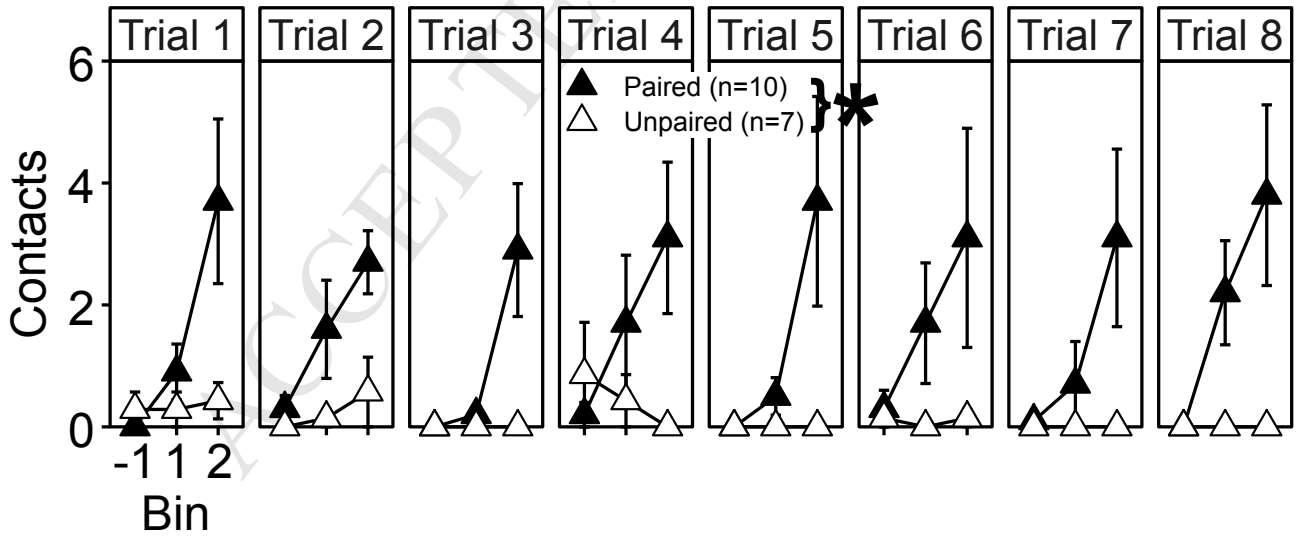




## A. Anticipatory Sipper Site Approach by Trial Within Conditioning Session 12

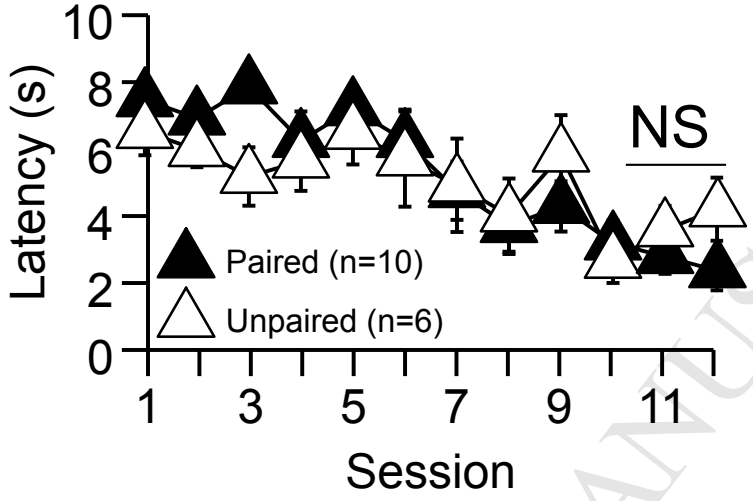



## B. Anticipatory Sipper Site Contacts by Trial Within Conditioning Session 12

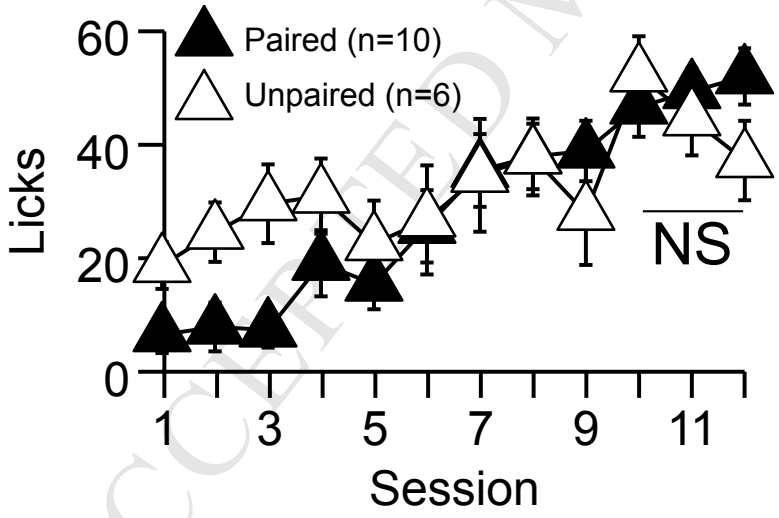


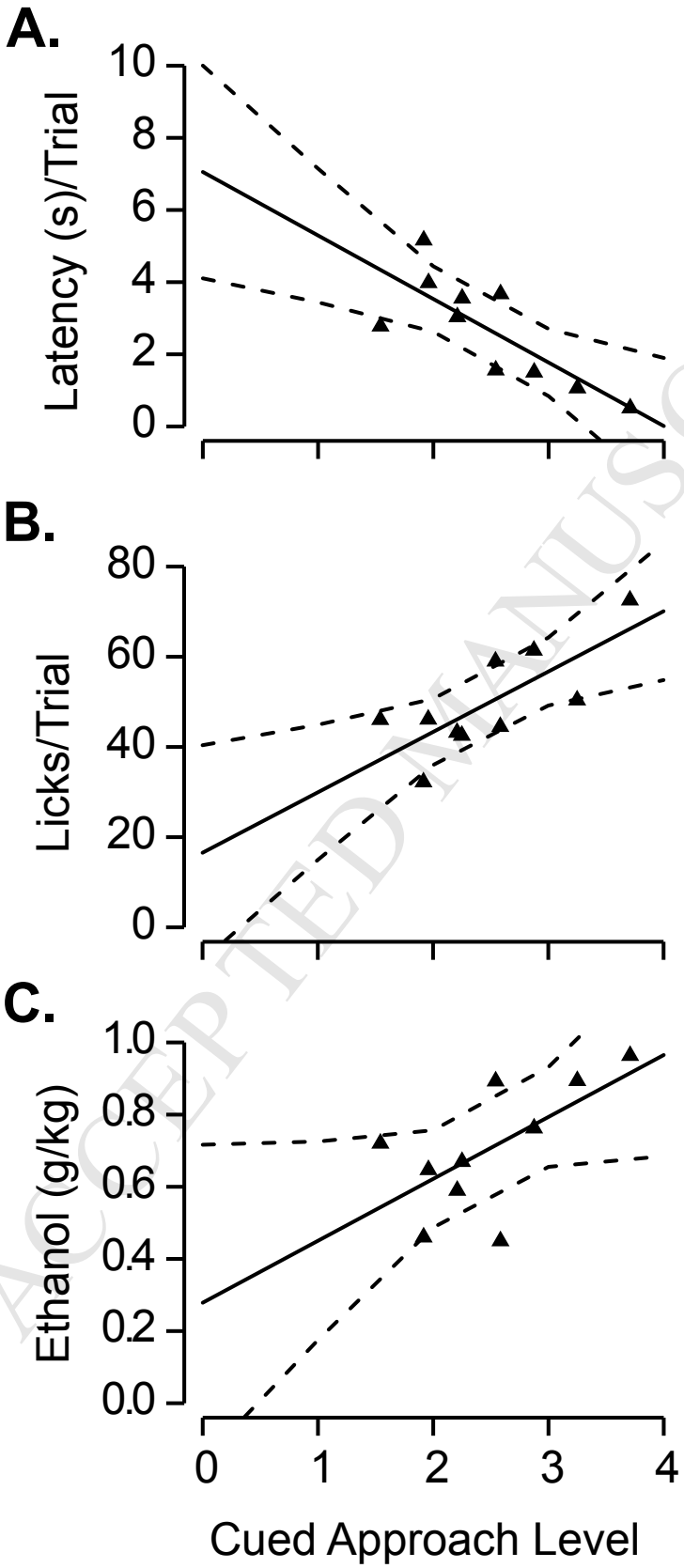
# Drinking Per Trial Across Conditioning

## A. Average Licking Latency Per Trial




## B. Average Total Licks Per Trial





Highlights

- Confirmed associative basis of cue-conditioned alcohol approach response in female rats
- The light cue elicited anticipatory alcohol seeking only when it was explicitly paired with alcohol access
- The alcohol sipper always gained the ability to elicit the initiation of alcohol drinking
- In the paired light cue-alcohol group, the vigor of alcohol seeking did not decrease within the conditioning session
- In both groups, the vigor of alcohol drinking decreased within the conditioning session