Supplemental Information

This supplemental file accompanies the article "Alcohol-associated antecedent stimuli elicit alcohol seeking in non-dependent rats and may activate the insula" by Cofresí, Grote, Le, Monfils, Chaudhri, Gonzales, & Lee.

Fos immunostaining & analysis procedures

Brains were sliced on a freezing-microtome and 30 µm coronal sections were taken from prefrontal cortex through midbrain, and sequentially separated into 6 series. Series 1-4 were cryoprotected and stored at -80°C. Sections from series 5 underwent Nissl staining to verify anatomical locations of adjacent immunostained sections. Sections from series 6 underwent c-Fos immunostaining. The primary antibody was rabbit c-Fos antibody (1:2500 dilution) (Santa Cruz Biotechnology, Dallas, TX, USA). After primary incubation (48 hr at 4°C), sections were rinsed in 0.1 M PBS and incubated with biotinylated secondary antibody, anti-rabbit (1:250 dilution) (Vector Laboratories, Burlingame, CA, USA), for 1 hour. Next, sections were rinsed with 0.1 M PBS and incubated with pre-formed avidin/biotin enzyme complex (ABC) (Vector Laboratories). After additional rinsing with 0.1M PBS, sections were color reacted using 3,3-diaminobenzidine (DAB) with nickel sulfate to visualize c-Fos. Sections were imaged using an Olympus BX61 bright-field light microscope (Waltham, MA, USA) at 10x magnification. Images of each brain region were captured using QCapture (QImaging, Surrey, BC, CA) with 2x2 binning. The resulting images were analyzed by creating circular counting frames in ImageJ (NIH, Bethesda, MD, USA). Counting frame placements were chosen based on Swanson's rat brain anatomy atlas (2004). The area of each circular counting frame was calculated to 0.15 mm^2. Fos+ cells were counted within these sampling regions (counting frames) by raters blind to experimental conditions.

Using Swanson's rat brain anatomy atlas (2004), the following structures were sampled for analysis (anterior-posterior coordinates given relative to bregma; AP): (1) orbitofrontal cortex (lateral and medial) (AP: +5.20 to +3.20 mm), (2) medial prefrontal cortex (prelimbic and infralimbic) (AP: +4.85 to +2.15 mm and +3.20 to +2.15 mm, respectively), (3) nucleus accumbens (shell and core based on Paxinos et al., 2009) (AP: +2.15 to +0.95 mm), (4) dorsal striatum (lateral and medial) (AP: +2.15 to +0.95 mm), (5) insula (anterior and posterior based on Shi & Cassell, 1998) (AP: +4.85 to -2.45 mm), (6) amygdala (basolateral and central complexes) (AP: -1.53 to -2.85 mm), and (7) substantia nigra pars compacta and ventral tegmental area (AP: -4.60 to -5.25 mm). The

location and number of sampling regions per atlas level per brain region analyzed are shown in **Supplemental Figure 1**.

Ingested doses across conditioning by NonLearners

Supplemental Figure 2 shows ingested doses across conditioning sessions for the 12 rats (6 Paired, 6 Unpaired) that failed to drink ≥ 0.30 g/kg/session across sessions 10-12—the original asymptote identified in Cofresí et al., 2018—despite drinking enough in the homecage to be retained for conditioning. Across conditioning sessions, these rats ingested doses consistently below 0.30 g/kg/session (session main effect: $F_{11, 110}$=5.66, p<0.001; group main effect: $F_{1, 10}$<1, NS; session x group interaction: $F_{11, 110}$<1, NS).

Consummatory sipper licking across conditioning by Learners & NonLearners

Sipper licking was measured directly using a lickometer. Data from Learners (rats that drank > 0.30 g/kg/session across conditioning sessions 10-12) and NonLearners (rats that drank ≤ 0.30 g/kg/session across conditioning sessions 10-12) were analyzed separately. For these analyses, “trial” refers to sipper presentations, which occurred at different times during sessions for the Paired and Unpaired groups. Equipment malfunction resulted in failure to record sipper licking during at least one session for 1 rat in group Unpaired in the Learner class, reducing sample size to 7 for these analyses.

In the Learner class, we found that across sessions, there was a decrease in the average latency to start drinking per trial ($F_{11, 154}$=19.582, p<0.001), and a corresponding increase in the average size of drinking bouts ($F_{11, 154}$=2.787, p<0.001) (**Supplemental Figure 3 A-B**). ANOVA detected that the decrease in latency and increase in bout size varied by group (session x group interaction: $F_{11, 154}$≥3.707, p<0.05). As was the case for ingested doses, however, groups appeared to differ only at session 1 and 8, and those differences were not statistically significant after Bonferroni correction for multiple comparisons. In keeping, there were no significant group differences by the end of conditioning (group effects, session effects, & group x session interactions over sessions 10-12: NS).

In the NonLearner class, we found that across sessions, there was no change in the average latency to start drinking per trial in either group (session main effect, group main effect, & group x session interaction: NS) and

that there was small increase in the average size of drinking bouts in group Paired but not group Unpaired (session main effect: $F_{11, 99}$=1.82, p=0.06; session x group interaction: $F_{11, 99}$=2.42, p<0.05) (**Supplemental Figure 3 C-D**). However, groups did not differ in average drinking bout size at any session (simple main effects of group within sessions: F<1, NS).

Habituation of orienting reaction to houselight illumination

The rat's overt attentional/orienting response to houselight illumination is to rear (scored orienting state). Data from Learners (rats that drank > 0.30 g/kg/session across conditioning sessions 10-12) and NonLearners (rats that drank ≤ 0.30 g/kg/session across conditioning sessions 10-12) were analyzed separately.

In the Learner class, we found that rats in groups Paired (n=9) and Unpaired (n=8) differed in habituation of the orienting reaction to houselight illumination over the course of conditioning depending on (group x session interaction: $F_{11, 165}$=2.17, p<0.02; group x trial phase: $F_{4, 60}$=4.84, p<0.002; session main effect: $F_{11, 165}$=14.72, p<0.001; trial phase main effect: $F_{4, 60}$=16.13, p<0.001; **Supplemental Figure 4 A**). Collapsing across sessions, orienting frequency per trial was similarly low between groups before houselight onset and during the first half of illumination (1st and 2nd 5 s bin post-onset) (simple effects of group within each trial phase: NS). However, orienting per trial during the last quarter of illumination (4th 5 s bin post-onset) was different: greater in group Unpaired than Paired (simple effect of group: $F_{1, 15}$=41.12, p<0.001). Collapsing across trial phases, orienting frequency per trial within each group changed across sessions (simple effects of session: $F_{11, 88}$≥3.40, p<0.001).

In the NonLearner class, we found that rats in groups Paired (n=6) and Unpaired (n=6) alike habituated their orienting reaction across sessions (session main effect: $F_{11, 110}$=4.58, p<0.001; trial phase main effect: $F_{4, 40}$=3.92, p<0.01; **Supplemental Figure 4 B**), but that the overall level of orienting was greater in group Unpaired (group main effect: $F_{1, 10}$=7.34, p<0.05).

Thus, orienting to houselight illumination was completely habituated in group Paired in both Learners and NonLearners. In group Unpaired, there appeared to be only partial habituation, but to the same level in both Learners and NonLearners suggesting that this behavior change may be unrelated to the consequences of

ethanol drinking. Instead, it may be related to the taste of ethanol or uncertainty about the timing of ethanol access.

Although we cannot say confidently that the persistent orienting in group Unpaired in the Learner class is related to the consequences of ethanol drinking, we were curious about the within-session dynamics of this behavior in the Learner class and whether the within-session patterns changed across key sessions in the experiment. Consequently, we analyzed orienting to houselight illumination (specifically, across trial phases -1, 1, 2, 3, and 4) on a per trial basis for 9 well-trained rats in group Paired and 8 well-trained rats in group Unpaired across conditioning sessions 6, 9, and 12, as well as the session before brain collection day (**Supplemental Figure 5 A-D**).

Collapsing trial phase, trial, and session, the level of orienting was lower in group Paired than Unpaired (main effect: $F_{1, 15}$=24.62, p<0.001). The pattern of orienting across trials varied across sessions (collapsing trial phase, trial x session interaction: $F_{21, 315}$=1.84, p<0.02), but differently depending on group (group x trial x session interaction: $F_{21, 315}$=1.88, p<0.02). Specifically, in group Paired, there was some trial-by-trial variation in orienting level and some between-session change in overall orienting level (collapsing trial phase, simple trial main effect: $F_{7, 56}$=5.69, p<0.001; simple session main effect: $F_{3, 24}$=4.36, p<0.05; simple trial x session interaction: $F_{21,168}$=1.39, NS). In general, within sessions, the level of orienting in group Paired was greater across trials 1-4 than 5-8 (collapsing trial phase and session, t_8=2.70, p<0.001), and the overall level of orienting decreased after session 6 (although collapsing trial phase and trial, sequential pairwise comparison using 2-tailed t-tests did not detect a statistically significant difference before or after Bonferroni correction, it is clear from the figure that the overall level of behavior in this group is near floor). In group Unpaired, there was within-session trial-by-trial variation in orienting level that was stable across sessions and no change in overall level between sessions (collapsing trial phase, simple trial main effect: $F_{7, 49}$=8.86, p<0.001; simple session main effect: $F_{3, 21}$=0.1822, NS; simple trial x session interaction: $F_{21,147}$=1.86, NS). Specifically, within sessions, the level of orienting in group Unpaired was greater across trials 1-4 than 5-8 (collapsing trial phase and session, t_7=5.79, p<0.001). Furthermore, orienting varied across trial phases within trials differently depending on trial (trial phase x trial interaction: $F_{28, 420}$=9.34, p<0.001), and this pattern of variation depended on group (group x trial phase x trial interaction: $F_{28, 420}$=4.21, p<0.001), but not session (trial phase x trial x session and

group x trial phase x trial x session interaction: F<1.5, NS). Collapsing session, group Paired and Unpaired alike exhibited different within-trial patterns across trials (Paired: $F_{28, 224}$=4.44, p<0.001; Unpaired simple trial phase x trial interaction: $F_{28, 196}$=6.94, p<0.001). In group Paired, there was within-trial variation in orienting level in trial 1 (collapsing session, simple trial phase main effect: $F_{4, 32}$=6.15, p<0.001) such there was a small and short-lived yet statistically significant increase in orienting level at houselight onset (collapsing session, trial phase -1 v. 1: t_8=3.88, p<0.005; trial phase -1 v. 2: t_8=0.41, NS). There was also some evidence for this within-trial orienting pattern in trial 3 and 4 (collapsing session, simple trial phase main effects: $F_{4, 32}$≥5.30, p<0.005), but the within-trial trial phase comparisons did not survive Bonferroni correction. The simple trial phase main effect in other trials was not significant. In group Unpaired, there was also within-trial variation in orienting level in trial 1 (collapsing session, simple trial phase main effect: $F_{4, 28}$=6.79, p<0.001) such that there was a modest statistically significant increase in orienting level at houselight onset that was sustained across the illumination period (collapsing session, trial phase -1 v. 1: t_7=5.97, p<0.001; trial phase -1 v. 2: t_7=8.50, p<0.001; trial phase -1 v. 3: t_7=3.31, NS after Bonferroni correction; trial phase -1 v. 4: t_7=4.5, p<0.005). The simple trial phase main effect in other trials was not significant.

We also separately analyzed scored orienting per trial within the session given on brain collection day (n=4/group) (**Figure 5 E**). The overall level of orienting was lower in group Paired than Unpaired, but not significantly so (main effect: $F_{1, 5}$=4.46, p=0.088). Unsurprisingly given the reduced sample size and low level of orienting behavior in general, none of the effects of interest achieved statistical significance (trial phase, trial, group x trial phase, group x trial, trial phase x trial, group x trial phase x trial: NS). Given that the previous analysis found trial phase x trial interaction effects and that the grand mean in the present analysis was significantly different from 0 (intercept term: $F_{1, 5}$=12.53, p<0.05), we tested the interaction model within groups. In group Paired, there was no within-subject effects (trial phase, trial, and trial phase x trial interaction: NS)—and the grand mean was not significantly different from 0 (intercept term: $F_{1, 2}$=4.48, NS). In group Unpaired, the grand mean was significantly different from 0 (intercept term: $F_{1, 3}$=12.07, p<0.05) and the main effect of trial was almost significant ($F_{7, 21}$=2.18, p=0.08). In general, orienting patterns exhibited by group Unpaired in **Figure 5 E** are similar to those exhibited in **Figure 5 B-D** whereas the level of orienting by group Paired appeared to be at floor.

Acquisition of conditioned sipper site approach reaction to houselight illumination

One of the rat's classic learned responses to a reward-predictive cue is anticipatory approach to the reward delivery site (here: scored ethanol sipper site approach state). Data from Learners (rats that drank > 0.30 g/kg/session across conditioning sessions 10-12) and NonLearners (rats that drank ≤ 0.30 g/kg/session across conditioning sessions 10-12) were analyzed separately.

In the Learner class, rats in groups Paired (n=9) and Unpaired (n=8) differed in development of the sipper site approach reaction to houselight illumination over the course of conditioning (group x trial phase x session interaction: $F_{22, 330}$=2.512, p<0.001) (**Supplemental Figure 6 A**). Sipper site approach frequency per trial varied by trial phase (5 s bin before houselight onset, 1st and 2nd 5 s bin post-onset) across sessions only for rats in the Paired group (trial phase x session interaction: $F_{22, 176}$=2.225, p<0.05). In these rats, sipper site approach per trial during 10 s of houselight illumination increased in frequency across sessions (simple effects of session: $F_{11, 88}$>=6.125, p<0.001) whereas approach per trial before houselight onset remained at floor across sessions (simple effect of session: NS). In contrast, sipper site approach frequency per trial remained at floor across trial phases and sessions for rats in the Unpaired group (trial phase, session, & trial phase x session interaction: NS).

In the NonLearner class, rats in group Paired (n=6) and Unpaired (n=6) alike failed to develop a sipper site approach reaction to houselight illumination over the course of conditioning (group, trial phase, and session main effects and interactions: NS) (**Supplemental Figure 6 B**).

Thus, the sipper site approach reaction to houselight illumination was acquired only by group Paired rats in the Learner class, indicating that this learned behavior depends not only on the relationship between ethanol availability and houselight cue presentation, but also on drinking enough ethanol per session to potentially experience some of its post-ingestive effects.

History of free-choice ethanol drinking and preference in the homecage

As in (Cofresí, et al., 2017), we used an intermittent, 24-hr two-bottle choice procedure to familiarize rats with unsweetened ethanol in the homecage. In brief, rats were provided with a bottle of 15% ethanol in tap water

(v/v; 15E) every MWF for 5 weeks starting the week after arrival to the laboratory. Ethanol and water bottle placement on top of the homecage alternated (left/right) across MWF. Two water bottles were provided on TTSS. Chow was always available. The dose of ethanol ingested by each rat was monitored. Drinking solution intake was measured as the mass difference in bottle weight pre- and post-session after correcting for spillage. The grams of solution ingested were then converted to g ethanol and ingested dose was expressed as g/kg body weight for each rat. For every 24 hr homecage drinking session, bottles on an empty control cage were used to measure loss due to evaporation and spillage and correct solution intake values across all subjects. Only rats drinking ≥ 1 g/kg/24 hr across the last week of ethanol access in the homecage were retained for conditioning. Homecage phase drinking data from rats that subsequently drank > 0.30 g/kg/session across conditioning sessions 10-12 (Learner class) and rats that drank ≤ 0.30 g/kg/session across conditioning sessions 10-12 (NonLearner class) were first analyzed separately to confirm that group Paired and Unpaired were matched on homecage two-bottle history.

In the Learner class, across homecage drinking sessions, rats in the Paired (n=9) and Unpaired (n=8) groups similarly drank an increasing percentage of their total daily fluid from the ethanol bottle (session main effect: $F_{14,210}$=12.75, p<0.001; group main effect and group x session interaction: NS) (**Supplemental Figure 7 A**). Ingested doses increased similarly between groups across homecage sessions (session main effect: $F_{14,210}$=10.11, p<0.001; group main effect and group x session interaction: NS) (**Supplemental Figure 7 B**). Across the last week of homecage ethanol access, collapsing group, Learner rats (n=17) were drinking 51 ± 5 % of their total fluid (mean ± sem) from the ethanol bottle, ingesting doses of 3.4 ± 0.3 g/kg per 24 hr session.

In the NonLearner class, across homecage drinking sessions, rats in the Paired (n=6) and Unpaired (n=6) groups similarly drank an increasing percentage of their total daily fluid from the ethanol bottle (session main effect: $F_{14,140}$=6.61, p<0.001; group main effect and group x session interaction: NS) (**Supplemental Figure 7 C**). Ingested doses increased similarly between groups across homecage sessions (session main effect: $F_{14,140}$=4.62, p<0.001; group main effect and group x session interaction: NS) (**Supplemental Figure 7 D**). Across the last week of homecage ethanol access, collapsing group, NonLearner rats (n=12), were drinking 40 ± 5 % of their total fluid (mean ± sem) from the ethanol bottle, ingesting doses of 2.61 ± 0.4 g/kg per 24 hr session.

We next analyzed homecage two-bottle choice data for evidence of any difference between Learner & NonLearner rats collapsing cue conditioning group (Paired/Unpaired). ANOVA detected that on average, rats in the Learner class ingested greater doses and drank more from the ethanol bottle as a percentage of their total daily fluid than counterparts in the NonLearner class (class main effects: $F_{1, 27} \geq 6.00$, $p<0.05$), but that drinking behavior increased across homecage sessions similarly within classes (session main effects: $F_{1, 27} \geq 3.33$, $p<0.001$; class x session interactions: $F_{14, 378}<2$, NS). Although neither ANOVA detected a class x session interaction, we remained curious whether Learners and NonLearners differed in initial or final level of preference for ethanol (indexed here by their % total daily fluid drank from ethanol bottle across sessions 1-3 or 13-15, respectively). To this end, we performed two 2-tailed t-tests. The t-test for the final preference level difference between classes was not significant before or after Bonferroni correction ($t_{27}=1.37$, NS). The t-test for the initial preference level difference between classes was marginally significant before Bonferroni correction ($t_{27}=2.02$, $p=0.053$, corrected-$p=0.106$).

References

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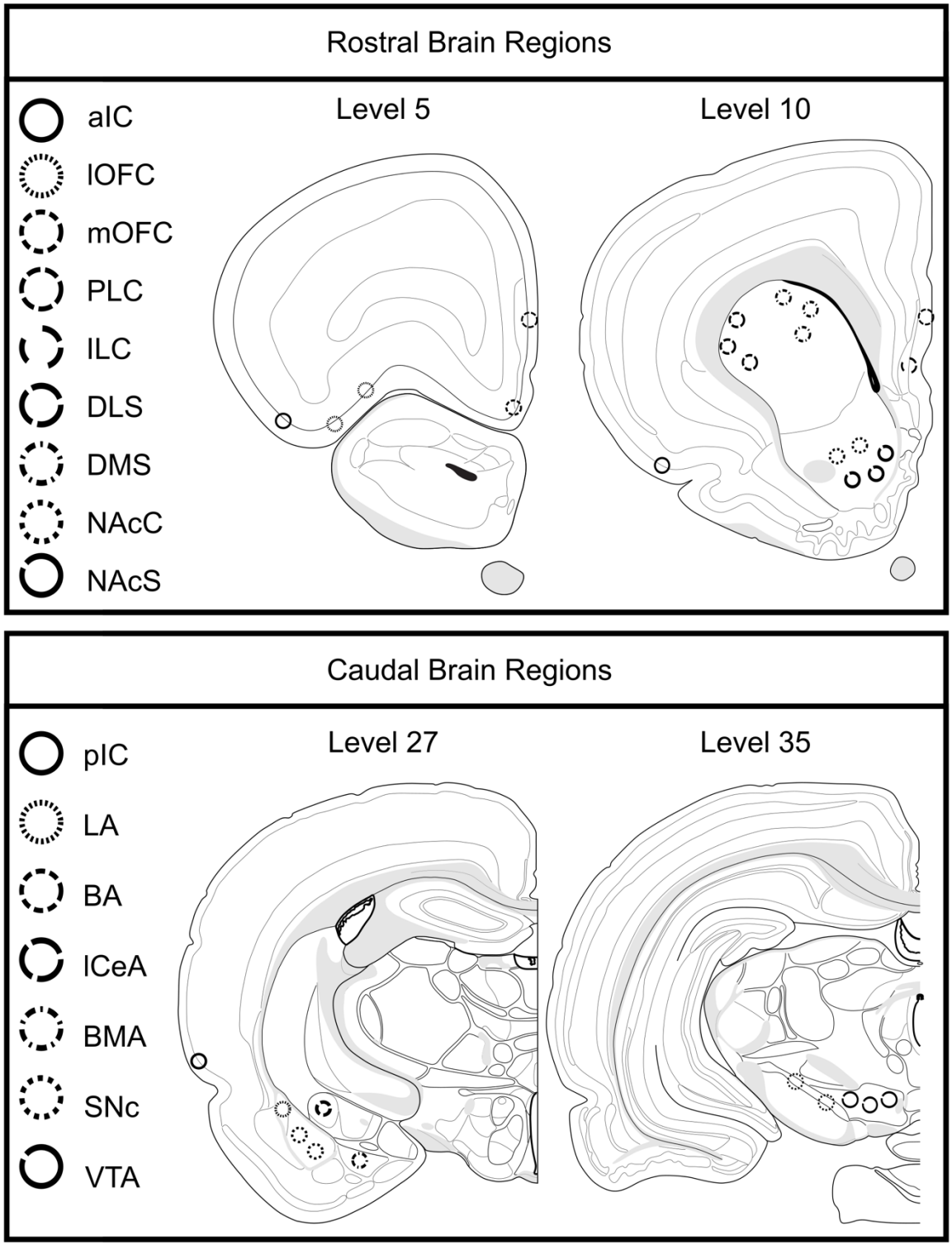
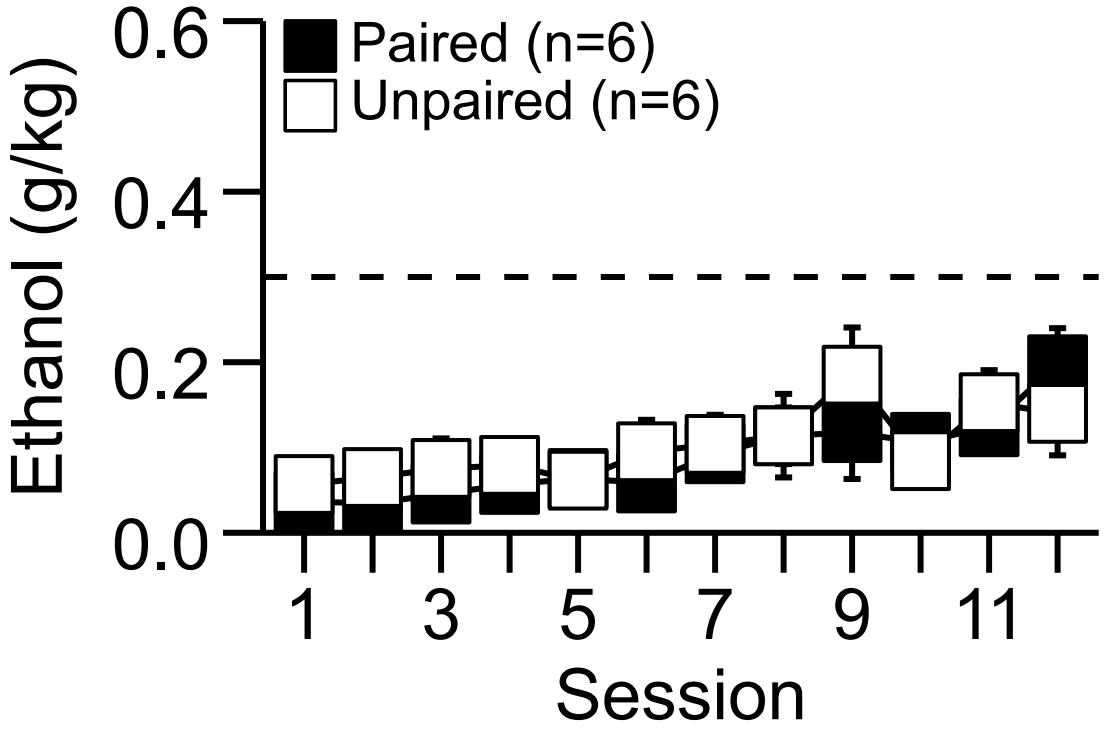
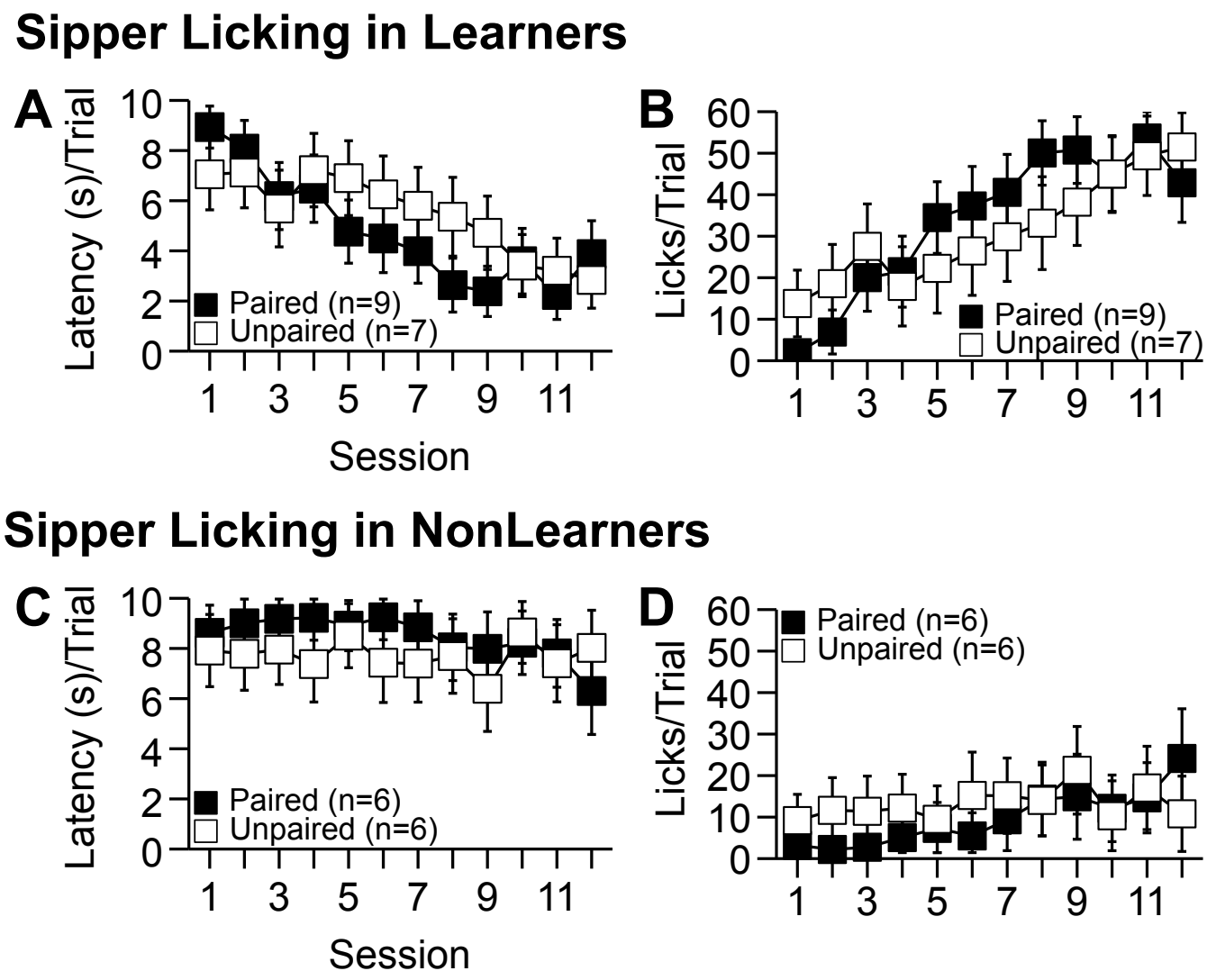


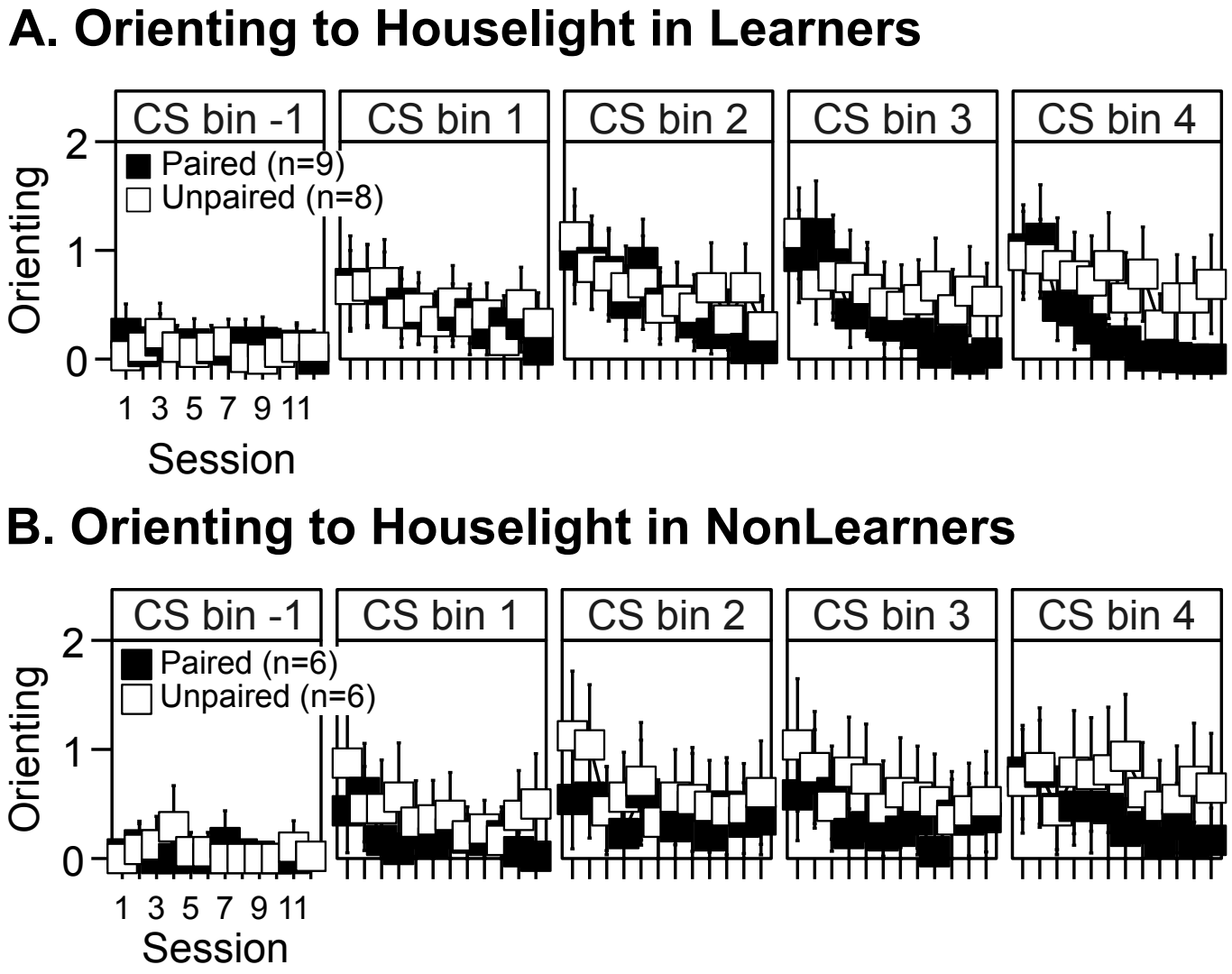
Supplemental Figure 1. c-Fos study. Location and number of Fos+ cell sampling regions (0.15 mm^2 each) per brain region per hemisphere shown on representative plates from Swanson's rat brain atlas. Refer to main and supplemental text Methods: Fos for details.



Supplemental Figure 2. Ingested doses across conditioning in NonLearners. Mean ± sem ingested ethanol doses across conditioning sessions for adult, male Long-Evans rats that ingested ≤ 0.30 g/kg/session (indicated by dashed horizontal line) across session 10-12 (viz., rats in the "NonLearner" class).

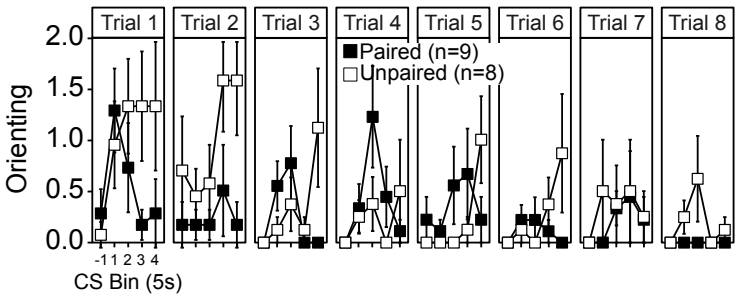


Supplemental Figure 3. Sipper licking across conditioning. A-B: Mean ± sem latency (s) to start licking (**A**) and total licks (**B**) per trial across conditioning sessions (8 trials/session) for adult, male Long-Evans rats that ingested ≥ 0.30 g/kg/session across session 10-12 (viz., rats in the "Learner" class). **C-D:** Mean ± sem latency (s) to start licking (**A**) and number of licks (**B**) per trial across conditioning sessions (8 trials/session) for adult, male Long-Evans rats that ingested ≤ 0.30 g/kg/session across session 10-12 (viz., rats in the "NonLearner" class). **A-D:** Licking was measured directly using a lickometer. Latencies (maximum latency was 10 s; omissions were recorded as maximum latency) were derived from lickometer data.

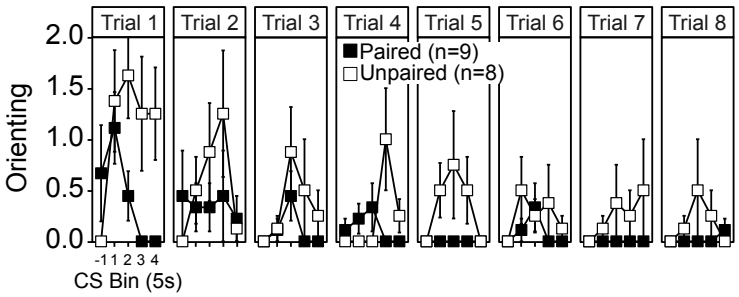


Supplemental Figure 4. Orienting to houselight across conditioning. Mean ± sem levels of orienting (rearing) to houselight illumination per trial across conditioning sessions (8 trials/session) paneled by trial phase (5 s bin before light onset followed by four bins post-onset; CS bins -1, 1, 2, 3, 4) for adult, male Long-Evans rats that (**A**) ingested ≥ 0.30 g/kg/session across session 10-12 (viz., rats in the "Learner" class) and (**B**) those that ingested ≤ 0.30 g/kg/session across session 10-12 (viz., rats in the "NonLearner" class). Orienting data (maximum response level was 4) were derived from offline manual videoscoring (see main text Methods: Behavior Measurement for details).

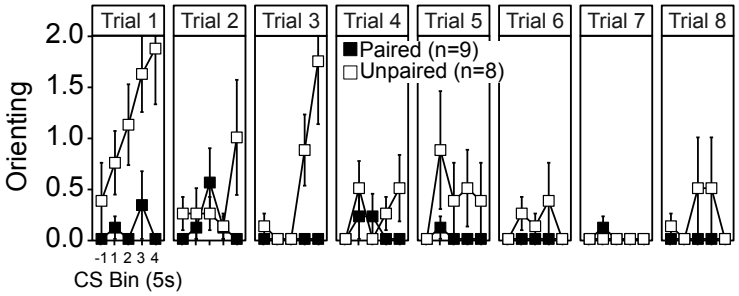
A. Session 6



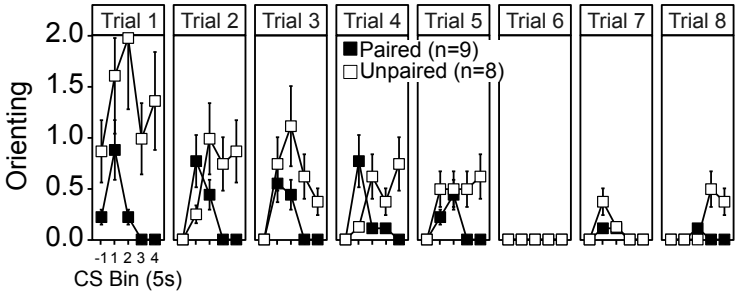
B. Session 9



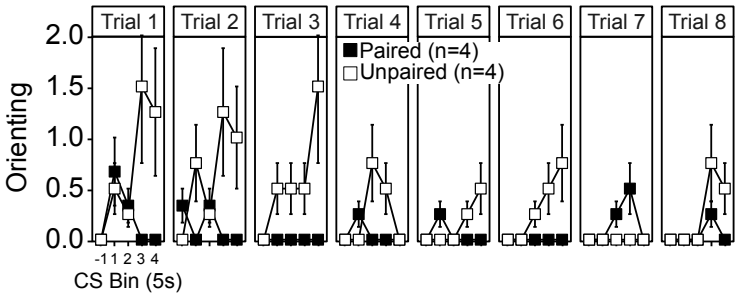
C. Session 12



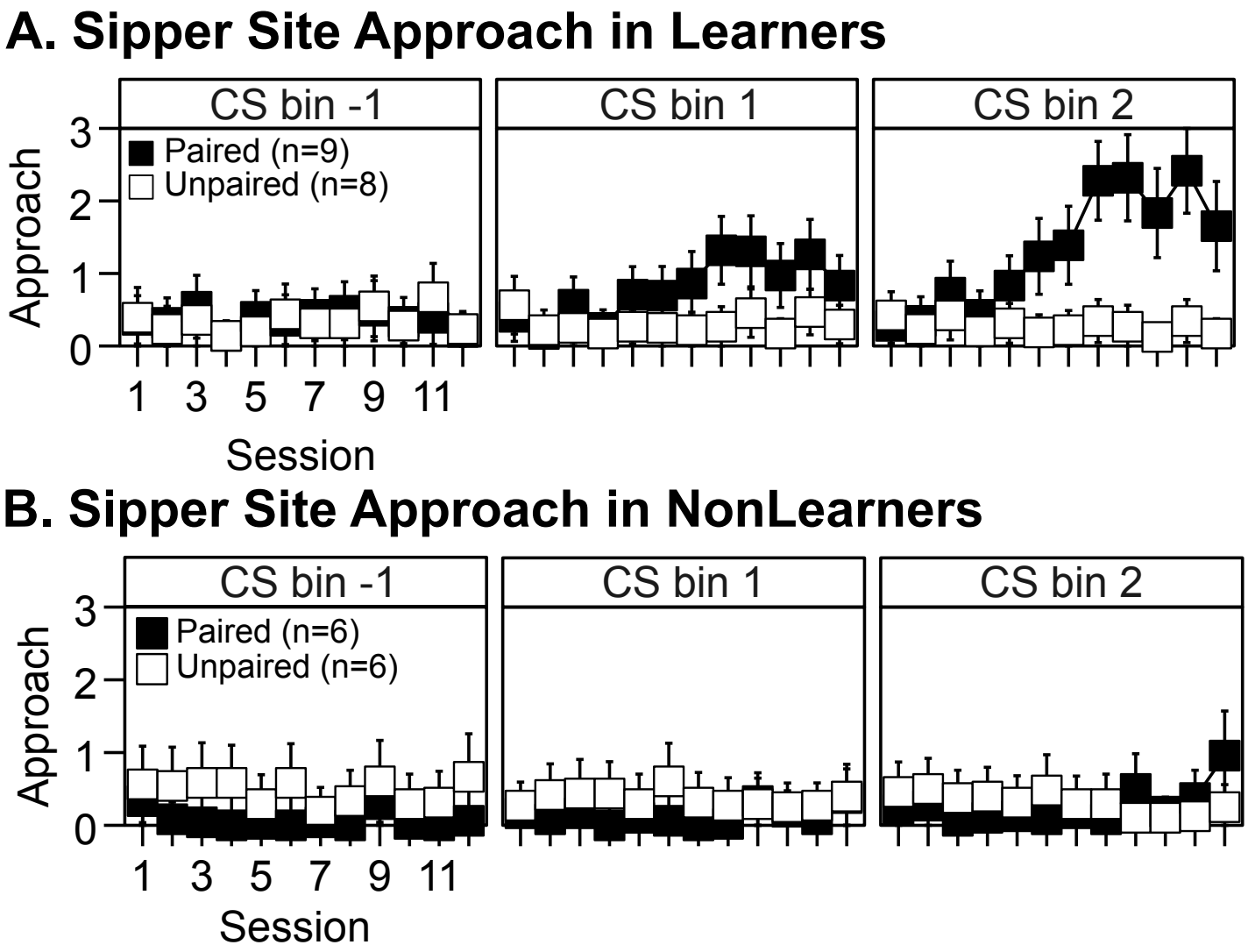
D. Day Before Brain Collection



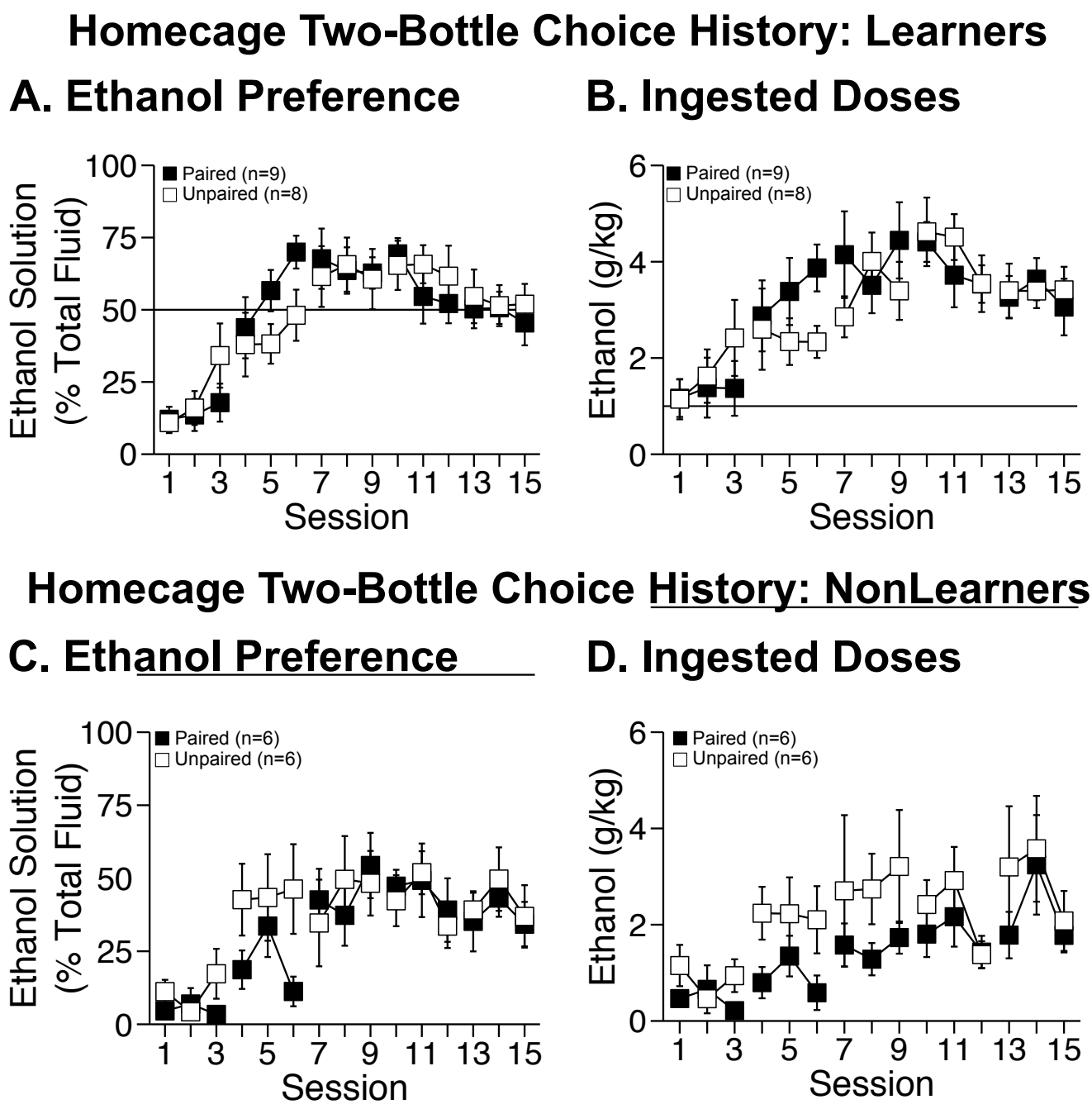
E. Brain Collection Day



Supplemental Figure 5. Orienting to houselight per trial. Mean ± sem level of orienting state in the 5 s before light onset (CS bin -1) and 20 s post-light onset (CS bins 1-4) paneled by trial (1-8) within select conditioning sessions for adult, male Long-Evans rats that ingested ≥ 0.30 g/kg/session across session 10-12 (viz., rats in the "Learner" class). Orienting data (maximum response level was 4) were derived from offline manual videoscoring.



Supplemental Figure 6. Ethanol sipper seeking across conditioning. Mean ± sem levels of sipper site approach per trial across conditioning sessions (8 trials/session) paneled by trial phase (5 s bin before light onset followed by two bins post-light onset, but pre-sipper onset; CS bins -1, 1, and 2) for adult, male Long-Evans rats that (**A**) ingested ≥ 0.30 g/kg/session across session 10-12 (viz., rats in the "Learner" class) and (**B**) those that ingested ≤ 0.30 g/kg/session across session 10-12 (viz., rats in the "NonLearner" class). Approach data (maximum response level was 4) were derived from offline manual videoscoring (see main text Methods: Behavior Measurement for details).



Supplemental Figure 7. Homecage two-bottle choice history. A: Mean ± sem fluid ingested from the ethanol bottle as a percentage of total fluid for adult, male Long-Evans rats that ingested ≥ 0.30 g/kg/session across session 10-12 (viz., rats in the "Learner" class). **B:** Mean ± sem ingested ethanol doses for the same. **C:** Mean ± sem fluid ingested from the ethanol bottle as a percentage of total fluid for adult, male Long-Evans rats that ingested ≤ 0.30 g/kg/session across session 10-12 (viz., rats in the "NonLearner" class). **D:** Mean ± sem ingested ethanol doses for the same. **B+D**: Horizontal line indicates *a priori* retention criterion for conditioning (1 g/kg/session across session 13-15).