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DID it or DIDn't it? Exploration of a failure to replicate binge-like alcohol-drinking in C57BL/6J mice☆

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ABSTRACT

We previously reported that commercially-sourced C57BL/6J (B6) male mice with a history of adult-onset binge-drinking exhibit anxiety-like behavior in early withdrawal, while the negative affective state incubates during protracted withdrawal in adolescent-onset binge-drinking males. As the results of such studies are potentially confounded by age-related differences in reactivity to environmental stress, we employed a 2-bottle-choice DID procedure (20 and 40% alcohol; 20 min habituation to the drinking cage) to examine the effects of binge-drinking on negative affect in male and female, adult and adolescent, B6 mice from our university colony. Unexpectedly, the mice in the initial experiment exhibited very low alcohol intake, with little sign of withdrawal-induced negative affect. This failure to replicate prompted us to examine how the duration of drinking cage habituation, the number of alcohol concentrations presented and the animal source might influence the propensity to binge-drink. Herein, we show that both male and female adult mice from our colony will binge-drink when allowed 45 min to habituate to the drinking cages, irrespective of whether mice are offered a choice between 2, 3 or 4 alcohol concentrations. Further, when drinking under 4-bottle-choice procedures (5, 10, 20 and 40% alcohol), adult-onset binge-drinking females exhibit robust negative affect in early withdrawal akin to that reported previously for adult males; however, the negative affective state persists for at least 30 days into withdrawal. Also unlike males, adolescent-onset binge-drinking females exhibit some signs of negative affect, as well as potentiated alcohol intake, in early withdrawal, which persist into later withdrawal. These latter data suggest that the age-related differences in the temporal patterning of the negative affective state produced by alcohol withdrawal may vary as a function of sex, which may have implications for understanding sex differences in the etiology of affective disorders and alcoholism co-morbidity.

1. Introduction

In humans, alcohol drinking typically commences during adolescence (ages 13–14), with binge-drinking being the most common pattern of alcohol intake (e.g., SAMHSA, 2010). Binge-drinking, as well as the incidence and prevalence of alcohol use disorders (AUDs), peaks in late adolescence/early adulthood (e.g., Chen et al., 2004; Harford et al., 2005; Martin and Winters, 1998). This is concerning as this age-range constitutes a critical period for the establishment of corticofugal connectivity within the extended amygdala neural circuit regulating emotional reactivity (e.g., Gogtay et al., 2004; Sowell et al., 2001; Spear, 2000a; Steinberg, 2005). As such, binge-drinking is theorized to be an important neurodevelopmental insult that impedes the resolution of a hyper-emotional state upon the transition from adolescence to adulthood (e.g., Guerri and Pascual, 2010; McClure and Pine, 2007; Miller et al., 2007). In support of this theory, AUDs exhibit a very high degree of co-morbidity with all affective disorders (e.g., SAMHSA, 2010). However, it is unclear from the epidemiological data which disorder precedes the other in comorbid individuals. Complicating the issue, AUDs, affective disorders and their comorbidity are sexually dimorphic in humans (e.g., Grant et al., 2004; Hasin et al., 2007; Johnston et al., 2008; Kessler et al., 2005; Merikangas et al., 2007) and this dimorphism appears in early adolescence (Fox and Sinha, 2009; Johnston et al., 2008; Sonne et al., 2003; Witt, 2007). The gender gap in heavy drinking

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is closing, particularly amongst youth (e.g., Johnston et al., 2008; Keyes et al., 2010) and clinical evidence indicates accelerated AUD and neuropsychiatric disease progression in women versus men (e.g., Hommer et al., 2001; Keller et al., 2010; Schuckit et al., 1998; Sharrett-Field et al., 2013). Thus, there is a pressing need for more basic animal research focusing on the interactions between the age of heavy drinking-onset and sex with respect to the manifestation of negative affect (c.f., Fox and Sinha, 2009; Guerri and Pascual, 2010).

Adolescence in rats and mice is approximately 2 weeks long, and occurs between postnatal days (PNDs) 35–48 (e.g., Spear, 2000b). Thus, the comparative study of adult and adolescent binge-drinking upon emotional reactivity requires animals to voluntarily consume sufficiently high levels of alcohol during a "single sitting" to produce BECs ≥80 mg% (see National Institute on Alcohol Abuse and Alcoholism, 2007) and to do so consistently over the course of at least 2 weeks. However, as highlighted in several recent reviews (c.f., Becker, 2017; Spear, 2018), the majority of the rodent literature regarding age-related differences in alcohol-affect interactions employed forced alcohol exposure procedures (i.e., experimenter-administered injections, oral infusions or vapor chamber delivery) or lengthy self-administration procedures. Nevertheless, as reported in humans (e.g., Bogin et al., 1986; Brown et al., 2008), the results of such animal studies indicate that adolescents are resilient to the behavioral and physiological "hangover" effects of early alcohol withdrawal (incl. anxiety-like behavior) that are typically manifested by alcohol-exposed adults.

Likewise, a limited number of voluntary binge-drinking studies using modified Drinking-in-the-Dark procedures (see Rhodes et al., 2005 for description of original procedure) have also reported an interaction between the age of drinking-onset and withdrawal in the expression of anxiety-like behavior in male C57BL/6J (B6) mice. More specifically, adult male B6 mice with a 2-week history of binge-drinking under 2-h-access, multi-bottle-choice procedures [e.g., 4-bottle-choice (4-BC): 5, 10, 20 and 40%; 3-bottle-choice (3-BC): 10, 20, 40% alcohol, v/v; bottles presented at 3 h into the dark phase of the circadian cycle], exhibit robust signs of anxiety-like behavior in several paradigms (light-dark shuttle box, marble-burying and forced swim test) when assayed 1 day following drinking cessation (e.g., Lee et al., 2016, 2017a,b, 2018a,b). Further, behavioral signs of anxiety-like behavior are lower or completely absent when adult-onset drinking males are tested in later withdrawal (i.e., 30 days post-drinking cessation). Such findings argued that the alcohol-induced neuroadaptations driving the negative affective state in adult-onset binge-drinkers resolve or normalize with the passage of time in withdrawal. In contrast, despite consuming more alcohol over the 2-week drinking period *and* despite exhibiting more signs of basal anxiety than their adult counterparts, male B6 adolescents exhibit no overt emotional anomalies when tested at 1 day withdrawal. However, when tested 30 days later, the adolescent-onset binge-drinkers manifest very robust behavioral signs of negative affect (e.g., Lee et al., 2017b, 2018a). Thus, the manifestation of negative affect incubates during protracted alcohol withdrawal in male B6 mice with a history adolescent-onset binge-drinking, supporting the hypothesis that adolescent-onset binge-drinking alters the neurodevelopmental trajectory of circuits governing emotionality.

One major drawback of our prior work is the study of commercially-sourced mice, as the results are subject to the interpretational confound of age-related differences in transportation/relocation stress. Another drawback relates to the sole focus on male subjects, as female rodents tend to binge-drink more alcohol than males (e.g., Melón et al., 2013). This report describes a primary, large-scale, experiment designed to replicate our prior interaction between binge-drinking history, the age of drinking-onset, and withdrawal in the manifestation of negative affect in male and female mice derived from a B6 colony established in our university vivarium (Experiment 1). For this, distinct cohorts of male and female littermates were tested for signs of negative affect on withdrawal days 1 or 30, under a similar behavioral test battery as that used in our prior studies of vendor-sourced, male mice (e.g., Lee et al., 2017b). Based on evidence that both adolescent and adult male mice binge-drinking under multi-bottle-choice procedures consume the majority of their daily intake from the sipper tubes containing 20 or 40% alcohol (e.g., Lee et al., 2016, 2017b), the mice in Experiment 1 were offered only 20 and 40% alcohol to facilitate study through-put. Further, to accommodate the large number of animals required of this study, mice were group-housed with same-sex littermates and then transferred singly to drinking cages, located in a non-colony procedural room, to determine individual alcohol intake. To expedite the timing of bottle presentation, mice were allowed 20 min to habituate to the drinking cages prior to alcohol presentation. As in our prior work (e.g., Lee et al., 2017b), the alcohol was offered for 2 h/day, beginning at 3 h into the dark phase of the circadian cycle, for 14 consecutive days prior to behavioral testing.

Appropriate for inclusion in this special edition on reproducibility and replication, the results of this primary experiment failed to replicate the levels of binge-drinking observed in our published work (e.g., Lee et al., 2017b) and, not surprisingly, we detected no signs of withdrawal-induced negative affect. As these completely negative outcomes were unexpected, additional follow-up studies were then conducted to understand the basis for this replication failure. As one procedural variable that differed from prior work was the number/range of alcohol concentrations presented, the first follow-up experiment (Experiment 2) determined how the number of alcohol concentrations presented (2, 3 or 4) influenced the amount of alcohol consumed by adult, male and female, mice from our UCSB colony. As mice in our prior studies (e.g., Lee et al., 2017b) were allowed a minimum of 45 min to habituate to the drinking cages prior to bottle presentation, a > 45 min habituation period was employed in Experiment 2. As all mice in Experiment 2 consumed levels of alcohol demonstrated previously to result in BACs ≥80 mg%. The second follow-up experiment (Experiment 3) then examined how animal source influences the expression of alcohol withdrawal-induced negative affect. As we have replicated age-related differences in the temporal manifestation of negative affect and excessive alcohol-drinking during withdrawal in male mice (e.g., Lee et al., 2016, 2017b), Experiment 3 employed females exclusively to begin to make head-way towards characterizing how withdrawal from binge-drinking influences emotionality in female subjects. Experiment 3 employed 4-bottle-choice procedures (5, 10, 20 and 40% alcohol), based on the findings of Experiment 2 indicating that this procedure engendered the highest alcohol consumption in female subjects.

Combined, the results of these three experiments provide additional insight into experiential factors that impinge upon the levels of binge-drinking in male and female B6 mice to impact the manifestation of negative affect during withdrawal.

2. Materials and methods

The Materials and methods section begins with an outline of the designs of, and rationales for, the three experiments summarized in this report. The first subsection also outlines the procedural time-lines of the experiments and the statistical approaches employed to analyze the data. The remaining subsections then detail the specific procedures employed in this study.

2.1. Experimental designs, rationales and procedural time-lines

2.1.1. Experiment 1: replication and extension of age-related differences in withdrawal-induced negative affect

Experiment 1 examined for the interactions between sex, binge-drinking history (2-week DID vs. water), age of drinking-onset (adolescent vs. adult) and withdrawal (1 vs. 30 days) upon negative effect in mice bred in our UCSB colony. In Experiment 1, mice underwent a 2-BC or water-drinking procedure for 14 consecutive days and then were tested for negative affect at their predetermined withdrawal period. The

average total alcohol intake (i.e., g/kg alcohol consumed in a 2-h period) over the 14-day drinking period was analyzed using a Sex × DID (water vs. 2-BC) × Age (adolescent vs. adult) ANOVA. The data from our behavior test battery were analyzed using a Sex × Drinking (DID vs. Water) × Age (adolescent vs. adult) × Withdrawal (1 and 30 days) ANOVAs. For both analyses, significant interactions were deconstructed and followed-up using corrected *t*-tests, when appropriate. In the cases where non-significant main effects or interactions were detected, the data were collapsed across the non-significant factor(s) and re-analyzed along the relevant factors.

2.1.2. Experiment 2: influence of the number of alcohol concentrations available upon alcohol intake in male and female UCSB mice

The study of binge-drinking-induced changes in emotionality requires that our UCSB-bred mice engage consistently in binge-drinking behavior in the first place. Thus, Experiment 2 determined whether or not the low levels of alcohol intake exhibited by the mice in Experiment 1 reflected the decision to employ at a 2-BC procedure, *in lieu* of a 3- or 4-BC drinking procedure or the employ of a shorter habituation period prior to daily bottle presentation. As adult mice tend to binge-drink less alcohol than adolescents (e.g., Lee et al., 2017b; Melón et al., 2013), Experiment 2 employed adult mice exclusively. A between-subjects design was employed in which different groups of adult male and female UCSB-bred mice were presented with alcohol under 2-BC, 3-BC or 4-BC procedures (see Sect. 2.2) for 14 consecutive days. The data for alcohol intake and our measures of negative affect were analyzed using a Sex × Bottle (2-BC, 3-BC, 4-BC) ANOVA and significant interactions were deconstructed prior to follow-up analyses. As conducted in Experiment 1 (see Sect. 2.4.1), the data were collapsed across the non-significant factor(s) and re-analyzed along the relevant factors.

2.1.3. Experiment 3: influence of animal source upon withdrawal-induced negative affect and subsequent binge-drinking expressed by female mice

The results of Experiment 2 pointed to the duration of the habituation period, rather than the number of bottles presented, as an important procedural variable regulating alcohol intake in adult UCSB mice. While the results of Experiment 2 demonstrated clearly that both male and female adult UCSB will engage in binge-drinking when allowed a minimum 45-min habituation period to the drinking-cages, it remained to be determined whether or not age-related differences in amount of binge-drinking, and its consequent effects on emotionality, might also vary with animal source. Thus, Experiment 3 compared the effects of withdrawal from binge-drinking between adult and adolescent B6 mice from The Jackson Laboratory or our UCSB colony. As the results of Experiment 1 could not inform as to how a binge-drinking history impacts emotionality in female mice, Experiment 3 employed female subjects exclusively. Based on the data from Experiment 2, Experiment 3 employed a 2-week, 4-BC procedure (5, 10, 20 and 40% alcohol, v/v) to maximize alcohol intake and consequent effects upon negative affect in early and later withdrawal. The data for alcohol intake were analyzed using a Source (JAX vs. UCSB) × Day (14 days) × Withdrawal (1 vs. 30 days) × Concentration (5, 10, 20 and 40% v/v) ANOVA, with repeated measures on the Days and Concentration factors. The behavioral data were analyzed using a Source (JAX vs. UCSB) × Age (adolescent vs. adult) × DID (water vs. 4-BC) × Withdrawal (1 vs. 30 days) ANOVA. The data were collapsed across non-significant factors prior to deconstruction of significant interactions, as appropriate. All data were analyzed using SPPS v.23.

2.2. Subjects

Male and female C57BL/6J (B6) mice were obtained from The Jackson Laboratory (Sacramento, CA). A subset of these animals (herein, referred to as JAX) were assayed for the effects of binge-drinking upon withdrawal-induced negative affect (see Sect. 2.3 and 2.4 below), while another subset was employed to establish a breeding colony of B6 mice in the Psychology Building vivarium (minimum of 20 breeding pairs) and generate research subjects (F2–5) naïve to transportation/relocation stress (herein, referred to as UCSB). As in our prior studies of male B6 mice (e.g., Lee et al., 2017b), the JAX adolescent and adult males and females arrived 1 week prior to testing to acclimate to the colony conditions and 12-h reverse cycle (lights off: 1000 h). The UCSB mice were maintained on a regular 12-h light cycle (lights on: 0700 h) until 1 week prior to testing, at which time they were relocated to the reverse cycle room such that the time allowed for acclimation to the reverse cycle was consistent with that of the JAX mice. All animals were housed in same-sex, age-matched groups of 4 per cage, with standard rat chow (Purina LabDiet) and water available ad libitum except during the 2-h alcohol-drinking period. All cages were lined with woodchip bedding. All procedures were approved by the Institutional Animal Care and Use Committee of the University of California Santa Barbara and were conducted in compliance with The Guide for the Care and Use of Laboratory Animals (2014).

2.3. Multi-bottle-choice Drinking-in-the-Dark (DID) procedures

Half of the animals from each experimental group were subjected to 14 consecutive days of binge-drinking under 1 of 3 different multi-bottle-choice DID procedures. Consistent with our prior adolescent work (e.g., Lee et al., 2017b), alcohol access was restricted to 14 days for all animals, which corresponds to the approximate duration of early-mid adolescence in mice (e.g., Spear, 2000a). Adolescent mice began drinking on PND 28–29, while adult mice began drinking on PND 56–58. Each day, animals were separated into individual drinking cages and allowed to habituate (see Section 2.1 for duration of habituation for each individual experiment). Beginning 3 h into the circadian dark cycle, binge-drinking (DID) mice were presented with sipper tubes containing different concentrations of alcohol. All DID mice in Experiment 1 were given simultaneous access to 2 bottles containing 20% and 40% alcohol (v/v; 2-BC). In Experiment 2, subsets of mice underwent the same 2-BC procedures, or were presented with either 3 bottles containing 10%, 20% and 40% alcohol (v/v; 3-BC; Lee et al., 2017a) or 4 bottles containing 5%, 10%, 20% and 40% alcohol (v/v; 4-BC) for 2 h (e.g., Lee et al., 2017b). In Experiment 3, 4-BC procedures were used exclusively. In all experiments, control animals were transported to the drinking room but received water only (Water). Daily alcohol consumption was calculated by weighing the bottles immediately before and after the 2-h drinking period and expressed as a function of the animal's body weight (g/kg), which was determined weekly. Throughout this report, “alcohol intake” refers to the amount of alcohol consumed (g/kg) in a 2-h period. In Experiment 1, submandibular blood samples were collected from a subset of alcohol-drinking animals on day 11 of drinking, immediately upon conclusion of the 2-h drinking period. Blood alcohol concentration (BAC) was determined using an Analox alcohol analyzer (model AM1, Analox Instruments USA, Lunenburg, MA).

2.4. Behavioral testing for negative affect

Our prior studies of JAX male mice indicated that adult-onset binge-drinking augments anxiety-like behavior during early (1–2 days) alcohol withdrawal, but that this effect dissipates by 30 days withdrawal. In contrast, adolescent-onset binge-drinking produces no observable effects upon negative affect in early withdrawal, but a robust increase in both anxiety- and depressive-like behavior during protracted withdrawal (e.g., Lee et al., 2016, 2017b). To replicate and extend these initial findings in Experimental 1, behavioral testing was conducted across withdrawal days 1 and 2 (WD1) and withdrawal days 30 and 31 (WD30), using a 2-day test battery, similar to that employed in our original study of binge-drinking-induced negative affect (Lee et al., 2015). At both time-points and starting at lights-out, testing for affect

began on Day 1 with an test for acoustic startle and pre-pulse inhibition of acoustic startle (see Sect. 2.4.1), which was followed by the novel object encounter test (see Sect. 2.4.2) and the forced swim test (see Section 2.4.3). On Day 2, testing began (again at lights-out) with the light-dark shuttle-box test (see Section 2.4.4), followed by the marble-burying test (see Section 2.4.5). All tests were conducted under standard ambient lighting and animals were tested in the aforementioned order in cohorts of 8, with assays spaced ~30 min apart, with no > 4 cohorts run per test day.

2.4.1. Acoustic startle

Testing was conducted in sound-attenuated startle chambers (SRLAB, San Diego Instruments, San Diego, CA), each consisting of a Plexiglas cylinder (3.8 cm diameter) mounted on a Plexiglas platform, with a high frequency loudspeaker (28 cm above the cylinder) producing all acoustic stimuli. The background noise of each chamber was 70 dB. A piezoelectric accelerometer, attached to the base, detected and transduced movements within the cylinder were detected and transduced by a piezoelectric accelerometer and then digitized and stored by a PC-type computer. As conducted previously (Szumlinski et al., 2005), six different trial types were presented: startle pulse (st110, 110 dB/40 ms), low prepulse stimulus given alone (st74, 74 dB/20 ms), high prepulse stimulus given alone (st90, 90 dB/20 ms), st74 or st90 given 100 ms before the onset of the startle pulse (pp74 and pp90, respectively) and no acoustic stimulus (i.e. only background noise was presented; st0). The st110, st0, pp74 and pp90 trials were applied 10 times, st74 and st90 trials were applied five times, and all trials were given in random order. The average inter-trial interval was 15 s (10–20 s) and the data for startle amplitude were averaged across the different stimulus trials for each mouse prior to statistical analyses. Elevated basal activity and increased startle amplitude were interpreted as reflecting anxiety-like behavior, while reduced pre-pulse inhibition of acoustic startle was interpreted as reflecting a sensorimotor gating deficit. This test was approximately 20 min in length and immediately following testing, mice were removed from the startle chambers, the chambers and cylinders were wiped down with 30% ethanol and mice were transported within the lab to a distinct testing room housing the novel object test chambers.

2.4.2. Novel object

To test reactivity to a novel object as an index of neophobia-related anxiety (e.g., Misslin and Ropartz, 1981), animals were placed in a black Plexiglas activity arena, measuring 46 cm long × 42 cm wide × 40 cm high. In the center of the arena was placed a novel, inedible, object (patterned ceramic candlestick holder; measuring ~6 cm in diameter × 12 cm high). A zone was designated around the novel object and was used to monitor the animals' interaction with the novel object during the 2-min trial using AnyMazeTM tracking software (Stoelting Co., WoodDale, IL, USA).The number of contacts and total time spent in contact with the novel object, as well as the total distance traveled within the activity arena, were recorded.

2.4.3. Porsolt Forced Swim Test

Each animal was placed into an 11-cm diameter cylindrical container and the latency to first exhibit immobility (defined as no horizontal or vertical displacement of the animal's center of gravity for ≥5 s), total time spent immobile, and the numbers of immobile episodes were monitored throughout the entire 6-min trial period using ANY-Maze™ video-tracking. In the forced swim test, decreased swimming/increased immobility or floating is conventionally interpreted as reflecting greater depressive-like behavior or "behavioral despair" as it is sensitive to pharmacological reversal by anti-depressant drugs (e.g., Porsolt et al., 1977). However, in prior work, we have observed an age-related effect of alcohol withdrawal in this assay, with adults and adolescents showing, respectively, decreased and increased immobility during withdrawal (e.g., Lee et al., 2017b, 2018a). Interestingly, the *reduced* immobility exhibited by adult binge-drinking mice temporally coincides with the manifestation of anxiety-like, but not depressive-like, behavior in other paradigms (e.g., Lee et al., 2017b, 2018a) and can be reversed by systemic treatment with the anxiolytic busperione (Lee et al., 2017a). Thus, we interpret the reduced immobility expressed by adult binge-withdrawn mice as reflecting an anxiety-like state (i.e., "panic"; Lee et al., 2017a). Conversely, adolescent-onset binge-drinking *increases* immobility in the forced swim test, coincident with reduced sucrose preference (Lee et al., 2017b) – a behavioral sign interpreted as reflecting anhedonia (see Katz, 1982). Thus, we interpret the increased immobility exhibited by adolescent-onset binge-drinking mice as reflecting a depressive-like state. Unfortunately, the limited available colony space at the time of testing precluded our ability to assay the mice in the present series of experiments for their sucrose preference. Upon completion of forced swim testing, animals were allowed to dry and then returned to the colony room to recuperate prior to testing the next day.

2.4.4. Light-dark shuttle box

Animals were placed into a polycarbonate box measuring 46 cm long × 24 cm high × 22 cm wide containing two distinct environments for a 15-min trial. Half of the box was white and uncovered, the other half black and covered, and these two environments were separated by a central divider with an opening. The animals were first placed on the dark side and the latency to enter the light side, number of light-side entries, and total time spent in the light-side of the shuttle box were recorded using ANY-Maze™ tracking software. The dependent measures were: the number of light-side entries, latency to first light-side entry, total time spent on the light side, and the total distance traveled. Decreased interaction with the light-side was interpreted as reflecting increased anxiety-like behavior (e.g., Crawley, 1985), while the distance traveled in the light-side provided an index of general locomotor activity. Immediately following completion of light-dark testing, the mice were transported, in their home cages, to another distinct behavioral testing room where marble-burying was assayed.

2.4.5. Marble-burying

In our paradigm, 12 square glass pieces (2.5 cm^2 × 1.25 cm tall) were placed in an empty home cage, lined with woodchip bedding, 6 at each end. The entire 20-min trial was recorded using ANY-Maze™ video-tracking and the latency to start burying the marbles, as well as the time spent burying were determined upon video playback by an observer blind to the history or sex of the mice using a stopwatch. The total number of marbles buried at the end of each trial was also recorded upon removal of the mouse from the testing cage. The dependent measures were: the number of marbles buried, latency to first begin burying, and total time spent burying. Increased burying behavior was interpreted as reflecting increased defensive anxiety-like behavior (Njung'e and Handley, 1991).

3. Results

3.1. Experiment 1: interactions between sex, alcohol-drinking history, age of drinking-onset and withdrawal on anxiety in UCSB-bred mice

3.1.1. Failure to reproduce binge-like levels of alcohol consumption under 2-BC conditions in UCSB mice

Initial analyses of the average total alcohol intake/2 h session across the 14 days of drinking under 2-BC procedures indicated a main Sex effect [$F(1,108) = 16.09$, $p < 0.0001$] and a main Age effect [$F(1,108) = 4.60$, $p = 0.03$]. There was no difference in the alcohol intake between the mice slated to be tested at the different withdrawal time-points, as indicated by no Withdrawal effect ($p = 0.19$) and no interactions between any of the factors (see Supplemental Table 1). As depicted in Fig. 1, females consumed more alcohol than males, irrespective of their age of drinking-onset, and adolescents consumed more

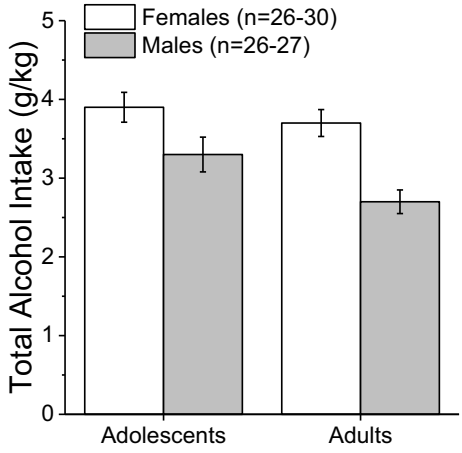

Fig. 1. Sex-related differences in alcohol intake are observed under 2-BC drinking procedures. A comparison of the average total alcohol intake exhibited by male and female adolescent and adult B6 mice bred at UCSB indicated greater alcohol intake overall, in females versus males and in adolescents versus adults. Data represent the means ± SEMs of the number of mice indicated in the figure.

Table 1
Summary of the BACs attained on Day 11 of drinking in Experiment 1, as well as the total alcohol intake exhibited by each drinking group over the 2-h drinking period. The data represent the means ± SEMs of the number of mice indicated.

	Adolescent		Adult	
	Male	Female	Male	Female
n	12	17	4	11
BAC (mg%)	33.5 ± 11.3	32.7 ± 6.6	29.3 ± 13.3	70.8 ± 12.5
Intake (g/kg)	3.7 ± 0.44	4.1 ± 0.4	3.1 ± 1.0	4.6 ± 0.3

alcohol than adults, irrespective of their sex. BACs were significantly, albeit weakly, correlated with intakes in the subset of mice tested ($r = 0.34$, $p = 0.02$; $N = 44$) and an analysis of group differences in BACs collected on Day 11 of drinking (see Table 1) failed to support any significant main effects of, or interaction between, the Sex and Age factors (see Supplemental Table 2). The average total alcohol intakes exhibited by all groups were either at, or below, those predicted from published correlational analyses to result in BACs ≥80 mg% (e.g., Cozzoli et al., 2014). Thus, while we replicated prior reports of sex- and age-related differences in alcohol intake by B6 mice (e.g., Melón et al., 2013; Strong et al., 2009), Experiment 1 failed to replicate binge-levels of alcohol intake under our 2-bottle DID procedures.

3.1.2. Low-dose alcohol-drinking does not alter acoustic startle

Initial analysis of group differences in the startle amplitude failed to indicate any main effect of, or interaction with, the DID factor (see Supplemental Table 3). Thus, the data were collapsed across this factor for re-analysis. As alcohol-drinking did not influence any of the dependent variables in the study, the detailed results are presented in the Supplemental material (Sect. S.1.1) and summarized in Supplemental Fig. 1A, B.

3.1.3. Low-dose alcohol-drinking does not alter prepulse inhibition of acoustic startle

Initial analysis of group differences in the inhibition of acoustic startle by the 74 and 90 dB prepulses revealed a significant DID × Age × Sex × Prepulse interaction [$F(1,196) = 6.97$, $p = 0.009$], but no main effect of Withdrawal (see Supplemental Tables 3 and 4 for complete results). Thus, the data were collapsed across the Withdrawal factor prior to deconstruction of the significant 4-way interaction along the Sex factor. In females, we detected a significant interaction between prepulse intensity and binge-drinking history [DID × Prepulse: $F(1,104) = 3.93$, $p = 0.05$], that was independent of the age of drinking-onset (no Age effects or interactions, p's > 0.10). However, when the data was collapsed across the Age factor, we did not detect any significant effect of alcohol-drinking history upon the prepulse inhibition produced by either the 74 dB [$t(106) = 1.72$, $p = 0.09$] or the 90 dB pre-pulse (*t*-test, $p = 0.69$) in female subjects (Fig. 2A). In males, we detected a significant 3-way interaction between drinking history, age of drinking-onset and prepulse intensity [$F(1,100) = 4.09$, $p = 0.04$]. Deconstruction of the interaction along the Age factor indicated no effect of alcohol-drinking in adolescent males (Fig. 2B, left; DID effect and interaction, p's > 0.40). We did detect a significant interaction between drinking history and pre-pulse intensity in adult males [$F(1,50) = 6.03$, $p = 0.02$]; however, post-hoc analyses failed to indicate any effect of drinking history on the percent inhibition produced by either pre-pulse stimulus (Fig. 2B, right; *t*-tests, df = 50, p's > 0.18).

3.1.4. Low-dose alcohol-drinking does not alter behavior in the novel object test

Initial analysis of the number of contacts, and the time spent in contact, with the novel object failed to indicate any group differences for these variables (data not shown; see Supplemental Tables 5 and 6). Initial analyses of the distance traveled indicated a main Age effect [$F(1,216) = 6.995$, $p = 0.009$] and an Age by Withdrawal interaction [$F(1,216) = 4.2$, $p = 0.04$; see Supplemental Table 7 for complete results]. As no significant Sex or DID effect was detected, the data were collapsed along both factors for re-analysis and the results of this analysis are presented in the Supplemental material (Section S.1.2; Supplemental Fig. 1C).

3.1.5. Low-dose alcohol effects upon behavior under light-dark shuttle-box procedures

Initial analysis of group differences in the latency to enter the light-side of the shuttle box failed to indicate any main effect of, or interaction with, the DID factor (see Supplemental Table 8 for complete results). Thus, the data were collapsed across this factor for re-analysis, the detailed results are presented in the Supplemental material (Section S.1.3) and significant findings summarized in Supplemental Fig. 1D.

Opposite our prediction, there was a trend for alcohol-experienced mice to spend *more* time in the light-side of the shuttle box [DID effect: $F(1,216) = 3.61$, $p = 0.06$], but this did not vary as a function of any of the other factors examined (see Supplemental Table 9 for complete results). Collapsing across all other factors, the alcohol-related difference in the time spent in the light-side reached statistical significance (Fig. 2C) [$t(215) = 1.99$, $p = 0.048$].

Initial analysis of the number of light-side entries indicated a significant 4-way interaction [$F(1,216) = 8.00$, $p = 0.005$; see Supplemental Table 10 for complete results]. Thus, the interaction was deconstructed along the Withdrawal factor. On WD1 (Fig. 2D, left), alcohol-drinking mice made more light-side entries than did water controls [DID effect: $F(1,110) = 4.63$, $p = 0.03$] and females made more entries than did males [Sex effect: $F(1,110) = 9.18$, $p = 0.003$]. No age-related effects nor interactions were detected for the number of light-side entries on WD1, although the 3-way interaction approached statistical significance (Fig. 2D, left) [$F(1,110) = 3.62$, $p = 0.06$]. Given that we predicted an age-related difference in anxiety during withdrawal, we deconstructed the 3-way interaction for WD1 along the Age factor. In adult mice (Fig. 2D, left, right subpanel), alcohol-drinking animals exhibited a *greater* number of light-side entries than water controls [DID effect: $F(1,50) = 8.01$, $p = 0.007$] and females entered the light-side more than males [Sex effect: $F(1.50) = 9.10$, $p = 0.004$]. Although inspection of Fig. 2D (left) suggested that the sex difference in light-side entries was driven primarily by the alcohol-drinking females, the Sex × DID interaction was not statistically significant ($p = 0.14$). In contrast, no group differences were observed in adolescent mice on

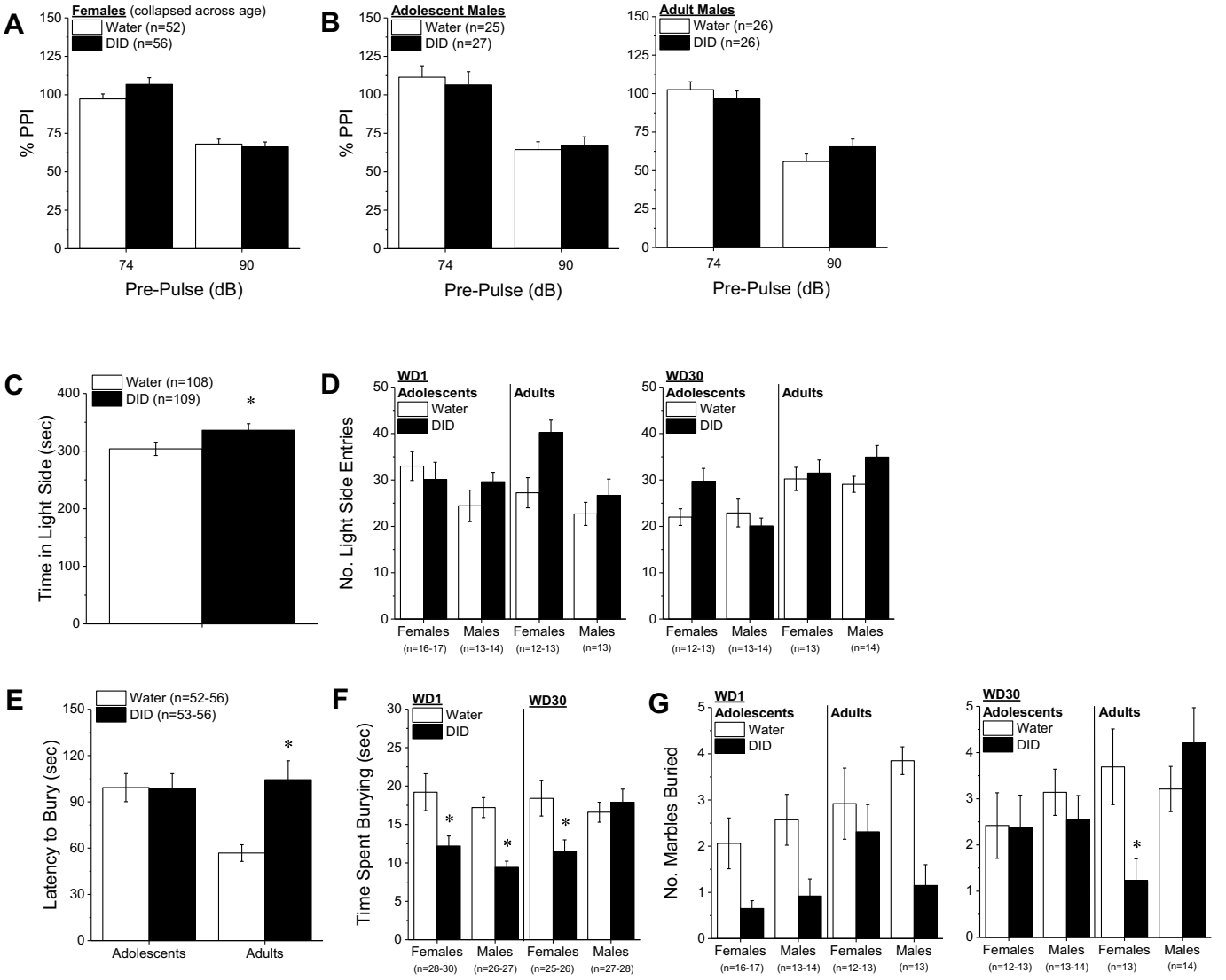

Fig. 2. Effects of low-dose alcohol-drinking upon sensorimotor-gating and indices of negative affect. (*A*) Despite detection of a significant interaction between alcohol-drinking history and pre-pulse, post-hoc analyses of the data for female mice bred at UCSB failed to detect any significant effect of alcohol-drinking upon the percent inhibition of acoustic startle (%PPI) elicited by either prepulse intensity. (*B*) Despite detection of a significant interaction between alcohol-drinking history, age of drinking onset, and prepulse, post-hoc analyses of the data for male mice bred at UCSB also failed to detect any significant effect of alcohol-drinking upon the %PPI exhibited by either adolescent (*left*) or adult (*right*) mice. (*C*) When collapsed across all other factors, animals with a history of low-dose alcohol consumption (DID) spent more time in the light-side of the light-dark shuttle box than did water-drinking controls (Water). (*D*) Summary of the 4-way interaction between our independent variables for the number of light side entries. (*E*) When collapsed across the withdrawal and sex factors, adult alcohol-drinking mice exhibited a longer latency to bury marbles than did their water controls, while no alcohol-related difference was observed in adolescent mice. (*F*) Summary of the interactions between sex, binge-drinking and age of drinking-onset for the time spent burying. (*G*) Summary of the 4-way interaction between our dependent variables for the number of marbles buried. Data represent the means ± SEMs of the number of mice indicated in the figure. *p < 0.05 vs. Water.

WD1 (Fig. 2D, left, left subpanel; DID × Sex ANOVA, all p's > 0.16). On WD30, the 3-way interaction was statistically significant (Fig. 2D, right) [DID × Sex × Age: $F(1,105) = 4.85$, $p = 0.03$]. Thus, we deconstructed this interaction along the Age factor and detected no group differences in adult mice on WD30 (Fig. 2D, right, left subpanel; DID × Sex ANOVA, all p's > 0.14). However, a significant interaction was observed for the adolescent animals (Fig. 2D left, right subpanel) [$F(1,51) = 4.54$, $p = 0.04$], which reflected significantly more light-side entries in female alcohol-drinking mice versus their water controls [$t(23) = 2.29$ $p = 0.03$], but no drinking effect in males (*t*-test, $p = 0.45$).

Taken together, the results of the light-dark shuttle-box test represent a failure to replicate an alcohol withdrawal-induced increase in anxiety-related behavior, as well as the age-related differences therein. While we did detect age- and/or alcohol-related differences in behavior in this assay, the direction of the observed effects were opposite those reported previously by our group (e.g., Lee et al., 2017b).

3.1.6. Failure to replicate withdrawal-induced anxiety-like behavior under marble-burying procedures

Initial analysis of the latency to begin burying marbles indicated significant main effects of Withdrawal [$F(1,216) = 4.24$, $p = 0.04$], which reflected an overall longer latency to bury in early versus later withdrawal (WD1: 100.01 ± 6.76 vs. WD30: 79.81 ± 6.87 s). We also observed a main alcohol-drinking effect [$F(1,216) = 5.81$, $p = 0.02$], as well as an interaction between alcohol-drinking history and the age of drinking-onset [$F(1,216) = 7.20$, $p = 0.008$] (see Supplemental Table 11 for complete results). As there were no significant interactions with the Withdrawal factor and no statistically significant effect of, or interactions with, the Sex factor (see Supplemental Table 11), the data

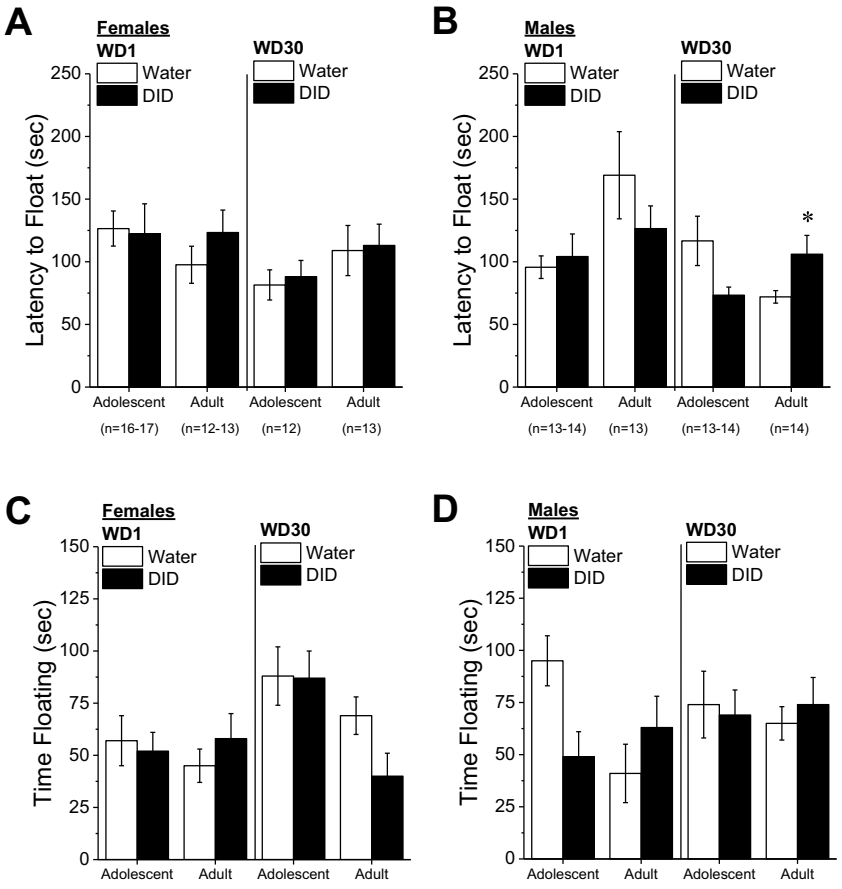

Fig. 3. Effects of low-dose alcohol intake upon behavior in the forced-swim test. (*A* and *B*) Depiction of the significant DID × Age × Sex × Withdrawal interaction observed for the latency to float in the forced swim test. (*A*) Follow-up analyses failed to detect any effect of alcohol-drinking in female mice on either withdrawal day 1 (WD 1) or 30 (WD30). (*B*) In males, the follow-up analyses indicated a significant DID × Age × Withdrawal interaction that reflected a longer latency to float in adult, alcohol-experienced mice on WD30. (*C* and *D*) Depiction of the modest 4-way interaction observed for the time spent floating in Experiment 1. (*C*) Follow-up analyses failed to detect any alcohol-drinking effect in female mice, while (*D*) an Age × DID interaction was observed in males that did not vary as a function of the withdrawal period. Data represent the means ± SEMs of the number of mice indicated in the figure. *p < 0.05 vs. Water.

for the latency to begin marble-burying were collapsed across these factors for re-analysis of drinking-related effects. Re-analysis supported a significant DID by Age interaction [$F(1,216) = 6.52$, $p = 0.01$], which reflected a *shorter* latency to bury by adult water controls, relative to their alcohol-drinking counterparts (Fig. 1E, [$t(103) = 3.54$, $p = 0.001$], but no alcohol-related differences in adolescent animals (Fig. 1E; *t*-test, $p = 0.97$).

Initial analysis of the data for the time spent burying indicated an overall alcohol-drinking effect [$F(1,216) = 9.36$, $p = 0.003$], which reflected a *shorter* time spent burying by alcohol-experienced versus water control mice [Water: 17.85 ± 0.93, n = 108 vs. DID: 12.77 ± 0.75, n = 109]. While sex differences were not detected for the latency to begin burying marbles (see above), there were significant or near-significant interactions with the Sex factor as indicated by the initial analysis of the data for the time spent burying (see Supplemental Table 11 for complete results). However, as initial analyses failed to indicate any age-related differences in the time spent burying (see Supplemental Table 12), the data were collapsed across this factor for re-analysis. Re-analysis indicated a significant interaction between sex and withdrawal [$F(1,216) = 19.20$, $p < 0.0001$], which varied, albeit modestly, with the drinking history of the mice [DID × Sex × Withdrawal: $F(1,216) = 3.66$, $p = 0.06$]. We also detected a near-significant interaction between drinking history and withdrawal for the time spent burying [$F(1,216) = 3.84$, $p = 0.05$] and we explored these interactions further by deconstructing this interaction along the Withdrawal factor. On WD1, alcohol-drinking mice spent *less* time burying than water controls, irrespective of sex (Fig. 2F, left) [DID effect: $F(1,110) = 21.64$, $p < 0.0001$; Sex effect and interactions, p's > 0.14]. However on WD30, the effect of drinking varied as a function of sex [DID × Sex interaction: $F(1,105) = 5.66$, $p = 0.02$], with female alcohol-drinking mice spending less time burying than their water controls (Fig. 2F, right) [$t(49) = 2.55$, $p = 0.01$], but no alcohol-related differences in males (*t*-test, $p = 0.56$).

Initial analysis of the total number of marbles buried revealed a complex interaction between all of our factors [Sex × Age × DID × Withdrawal: $F(2,216) = 6.83$, $p = 0.01$; see Supplemental Table 13 for complete results]. Thus, the data were deconstructed along the Withdrawal factor to examine for time-dependent changes in behavior. On WD1, we detected less marble-burying, overall, in alcohol-drinking mice versus their water controls (compare open vs. closed bars in Fig. 2G, left) [DID effect: $F(1,110) = 21.26$, $p < 0.0001$] and in adolescent versus adult mice (compare right vs. left panel in Fig. 2G, left) [Age effect: $F(1,110) = 8.48$, $p = 0.004$; Adolescents: 1.53 ± 0.24, n = 60 vs. Adults: 2.55 ± 0.30, n = 51]. However, no significant interactions were observed between any of the factors on WD1 (Fig. 2G, left; DID × Age × Sex ANOVA, all p's > 0.10). In contrast, a significant 3-way interaction was observed for the number of marbles buried on WD30 (Fig. 2G, right) [$F(1,105) = 5.06$, $p = 0.03$]. Deconstructing this interaction along the Age factor failed to indicate any effects of our factors in adolescent subjects (Fig. 2G, right; DID × Sex ANOVA, all p's > 0.45), while a significant DID by Sex interaction was observed for adult mice (Fig. 2G, right) [$F(1,53) = 7.02$, $p = 0.01$], which reflected significantly lower marble-burying in female alcohol-drinking mice, relative to their water controls [$t(24) = 2.61$, $p = 0.02$], but no alcohol-related effect in adult male mice (*t*-test, $p = 0.28$).

Taken together, the results of the marble-burying test also represent a failure to replicate an alcohol withdrawal-induced increase in anxiety-related behavior, as well as the age-related differences therein. As observed for the light-dark shuttle box test (Section. 3.1.4), we did detect age- and alcohol-related differences in marble-burying behavior, however, the direction of the observed effects were opposite those reported previously by our group (e.g., Lee et al., 2017b).

3.1.7. Some replication of withdrawal-induced negative affect under forced swim procedures in UCSB mice

Initial analysis of the latency to first exhibit floating behavior in the forced swim test indicated a significant 4-way interaction [$F(1,210) = 4.80$, $p = 0.03$; see Supplemental Table 14 for complete results]. Deconstruction of the interaction along the Sex factor failed to indicate any significant main effects or interactions in female subjects (Fig. 3A; DID × Age × Withdrawal ANOVA, all p's > 0.10). In contrast, a significant 3-way interaction was detected in males [$F(1,104) = 6.82$, $p = 0.01$]. Deconstructing this interaction along the Withdrawal factor indicated that adult males took significantly longer to float than did adolescent males on WD1 [Age effect: $F(1,50) = 5.22$, $p = 0.03$], but there was no main DID effect or interaction detected in early withdrawal (Fig. 3B, left; p's > 20). On WD30, a significant DID by Age interaction was detected [$F(1,53) = 8.32$, $p = 0.006$]. Relative to their water controls, this interaction reflected a shorter and longer latency to float, respectively, in adult and adolescent alcohol-drinking mice on WD30 (Fig. 3B, right) [for adolescents: $t(25) = 2.03$, $p = 0.053$; for adults: $t(25) = 2.09$, $p = 0.047$]. These data constitute a replication of our prior work (e.g., Lee et al., 2017b, 2018a) and indicate that even low-dose alcohol consumption can alter behavior in the forced swim test.

While the data for the latency to float replicated our prior work in male mice (e.g., Lee et al., 2017b, 2018a), we failed to detect any effect of drinking upon the number of immobile episodes in the forced swim test (all p's > 0.40; Supplemental Table S15). Thus, the data were collapsed along the DID factor for re-analysis and the data are presented in the Supplemental materials (Sect. S1.4).

However, a modest 4-way interaction was noted for the time spent floating [$F(1,215) = 3.89$, $p = 0.05$; see Supplemental Table 16 for complete results]. As conducted for the latency to float, we constructed this interaction along the Sex factor and follow-up analyses of the data from females indicated a significant Age effect [adolescents > adults; $F(1,107) = 4.84$, $p = 0.03$], a significant Withdrawal effect [WD30 > WD1; $F(1,107) = 4.69$, $p = 0.03$], but no DID effect or interactions (Fig. 3C). A significant DID by Age interaction was observed in males [$F(1,107) = 4.89$, $p = 0.03$], and while inspection of Fig. 3D suggested that this interaction was driven by the data from WD1, the DID by Age interaction did not vary significantly as a function of withdrawal (3-way ANOVA, $p = 0.14$).

Taken together, the results of the forced swim test replicate, but only partially, age-related differences in alcohol withdrawal-induced changes in negative affect.

3.2. Experiment 2: effect of varying the number of alcohol concentrations available upon alcohol intake by male and female UCSB mice

Experiment 2 examined total alcohol intake under 2-BC (20 and 40% v/v), 3-BC (10, 20 and 40% v/v) or 4-BC (5, 10, 20 and 40% v/v alcohol) in adult male and female mice bred in-house at UCSB. As depicted in Fig. 4A and B, the total daily alcohol intake exhibited by UCSB mice fluctuated somewhat across the 14-day drinking period [Day effect: $F(13,507) = 14.32$, $p < 0.0001$] and, overall, females consumed more alcohol than males [Sex effect: $F(1,39) = 18.10$, $p < 0.0001$; Sex × Day: $p = 0.81$]. While intake tended to be the highest under 4-BC procedures, neither the amount nor the pattern of intake varied significantly as a function of procedure [Procedure effect: $F(2,39) = 3.13$, $p = 0.06$; Procedure × Day: $p = 0.08$; Sex × Procedure × Day: $p = 0.33$; see Supplemental Table 17 for complete results).

As expected based on the time-course data, an analysis of the average total alcohol intake exhibited by the mice over the 14-day drinking period indicated higher alcohol consumption in female versus males (Fig. 4C) [Sex effect: $F(1,39) = 18.10$, $p < 0.0001$]. Although inspection of Fig. 4C suggested that the alcohol intake of females increased linearly as a function of the number of alcohol concentrations available, the interaction between sex and procedure was shy of statistical significance (Sex × Procedure: $p = 0.06$; see Supplemental Table 18). As the alcohol intake under 2-BC procedures exhibited by both male and female mice in Experiment 2 was above that predicted to result in BACs ≥80 mg%, these data argue that the low alcohol intake exhibited by the UCSB mice in Experiment 1 did not simply reflect the employ of 2-BC drinking procedures, but rather likely reflected an insufficient habituation to the drinking cages prior to bottle presentation. However, because (1) alcohol consumption exhibited by the female mice in Experiment 2 tended to increase as a function of the number of alcohol concentrations presented and (2) our original studies of age-related differences in alcohol withdrawal-induced negative affect in males were conducted under a 4-BC procedure (Lee et al., 2016, 2017b), a 4-BC procedure was employed in Experiment 3 to engender the highest possible alcohol intake in female mice prior to testing for withdrawal-induced changes in negative affect and subsequent alcohol consumption.

3.3. Experiment 3: interactions between animal source, alcohol-drinking history, age of drinking-onset and withdrawal on anxiety in female mice

3.3.1. Animal source does not significantly affect alcohol intake under 4-BC procedures

An examination of the dose-response function for the average alcohol intake exhibited by female, adult and adolescent, mice indicated a significant Age × Concentration interaction [$F(3,183) = 14.67$, $p < 0.0001$]. However, we failed to detect any significant effect of, or interactions with, the Source factor with respect to alcohol intake (see Supplemental Table 19 for complete results). As depicted in Fig. 5A, JAX and UCSB females exhibited equivalent alcohol consumption, irrespective of their age of drinking-onset. Collapsing across Source, a comparison of alcohol intake at each concentration indicated that adolescent females consumed more 40% alcohol (v/v) than their adult counterparts [$t(63) = 4.26$, $p < 0.0001$], while there were no significant age-related differences at the other concentrations tested (*t*-tests, all p's > 0.08). These findings extend to females our prior work in males, indicating that adolescent mice consume more high-dose alcohol than adult mice under our 4-BC DID procedures (e.g., Lee et al., 2017b). Furthermore, the average total alcohol consumption exhibited by the female mice in this study exceeded 4 g/kg in a 2-h period, with adolescent females consuming nearly 1 g/kg more alcohol than their adult counterparts (Fig. 5B) [Age effect: $F(1,64) = 10.34$, $p = 0.002$; Source effect and interaction, p's > 0.45; see Supplemental Table 20 for complete results]. Thus, all of the female mice in Experiment 3 were engaged in high levels of binge-drinking behavior prior to testing for behavioral signs of negative affect and subsequent alcohol consumption.

3.3.2. Replication of age-related differences in alcohol withdrawal-induced anxiety in female B6 mice under light-dark shuttle-box procedures

The influence of animal-source upon anxiety-like behavior in the light-dark shuttle box depended upon the dependent variable examined. For example, no group differences were apparent regarding the latency to enter the light-side of the shuttle-box (data not shown; see Supplemental Table 21 for complete results).

However, an age-related difference was observed for the effect of alcohol withdrawal upon the number of light-side entries exhibited by female mice in Experiment 3 [Age × DID × Withdrawal: $F(1,130) = 27.80$, $p < 0.0001$]. Although UCSB females made fewer light-side entries, overall, than did JAX mice (data not shown) [Source effect: $F(1,130) = 10.72$, $p = 0.001$], animal source did not significantly affect age-related differences in withdrawal-induced anxiety as determined by this measure [Source × Age × DID × Withdrawal, $p = 0.79$; see Supplemental Table 22 for complete results]. As such, the data were collapsed across the Source factor for follow-up analyses and the interaction was deconstructed along the Withdrawal factor. On WD1 (Fig. 6A, left), we observed a significant DID × Age interaction [F

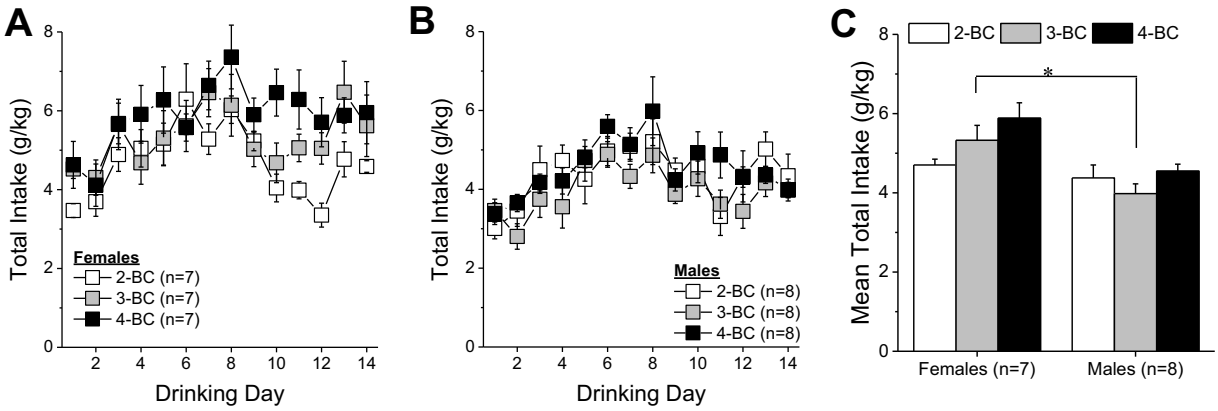

Fig. 4. Number of alcohol concentrations presented does not significantly impact total alcohol consumption by either male or female, adult, UCSB mice. Depiction of the time-course of total alcohol intake as a function of the number of bottles presented in female (*A*) and male (*B*) mice (BC = bottle-choice; 2-BC = 20 and 40% alcohol; 3-BC = 10, 20 and 40% alcohol; 4-BC = 5, 10, 20, 40% alcohol). (*C*) Depiction of the sex difference in total alcohol intake, across the different drinking paradigms. Data represent the means ± SEMs of the number of mice indicated in the figure. *Denotes main Sex effect ($p < 0.05$).

(1,65) = 10.85, p = 0.002], that reflected fewer light-side entries in water-drinking adolescents versus both their alcohol-drinking counterparts [t(31) = 2.30, p = 0.03] and water-drinking adult mice [t(31) = 2.62, p = 0.01]. In contrast, the number of light-side entries exhibited by alcohol-drinking adult females was lower than that of their water-drinking adult controls [t(32) = 2.17, p = 0.04]. On WD30 (Fig. 6A, right), we also observed a significant DID × Age interaction [F(1,64) = 14.16, $p < 0.0001$], however, this interaction reflected significantly fewer light-side entries in adolescent-onset alcohol-drinking mice, relative to both their water-drinking counterparts [t(32) = 5.35, $p < 0.0001$] and mice with a history of adult-onset alcohol-drinking [t(30) = 3.17, p = 0.004]. No significant age-related difference was detected for the number of light-side entries between adult- and adolescent-onset water-drinking mice (*t*-test, p = 0.07).

The pattern of group differences in the time spent in the light-side of the shuttle box (Fig. 6B) was similar to that observed for the number of light-side entries provided above, although the animal source exerted a more modest overall effect upon the time spent in the light-side (UCSB < JAX; data not shown) [F(1,130) = 3.88, p = 0.05]. Again, we detected a significant interaction between the DID, Age and Withdrawal factors for this variable [F(1,130) = 12.92, $p < 0.0001$] that was independent of animal source (4-way interaction, p = 0.11; see Supplemental Table 23 for complete results). Thus, the data were collapsed along the Source factor for follow-up analyses and the interaction deconstructed along the Withdrawal factor. On WD1 (Fig. 6B, left), no significant group differences were detected in the time spent in the light-side, although adult alcohol-drinking mice spent the least amount of time of all the groups tested. However, on WD30 (Fig. 6B, right), a significant Age × DID interaction was detected [Age × DID: F(1,65) = 13.10, p = 0.001], which reflected significantly less time spent in the light-side by adolescent-onset alcohol-drinking mice versus their water controls [t(32) = 4.53, $p < 0.0001$], with no alcohol effect observed in adult-onset females (*t*-test, p = 0.26).

Together, these data from the light-dark shuttle-box test argue that despite behavioral indices of higher overall anxiety in UCSB-bred mice, animal source does not significantly impact the age-related difference in the temporal patterning of alcohol withdrawal-induced anxiety-like behavior in female mice. Importantly, as reported for males (e.g., Lee et al., 2016, 2017b), females with an adult-onset binge-drinking history exhibit behavioral signs of anxiety-like behavior during early withdrawal that dissipate with the passage of time. In contrast, anxiety-like behavior incubates with the passage of time during alcohol withdrawal in females with a prior history of adolescent-onset binge-drinking.

3.3.3. *Partial replication of age-related differences in alcohol withdrawal-induced anxiety in female B6 mice under marble-burying procedures*

Analysis of the latency to marble-bury indicated an age-related difference in the effect of alcohol withdrawal (Fig. 7A) [Age × DID × Withdrawal: F(1,130) = 7.17, p = 0.009], but no effect of, or interactions with, the Source factor (all p's > 0.10; see Supplemental Table 24 for complete results). Thus, the data were collapsed across the Source factor prior to deconstruction of the 3-way interaction along with the Withdrawal factor. On WD1 (Fig. 7A, left), a significant DID × Age interaction was noted [F(1,65) = 11.92, p = 0.001], which reflected a shorter latency to marble-bury in alcohol-drinking adults versus both their water-drinking controls [t(32) = 7.10, $p < 0.0001$]

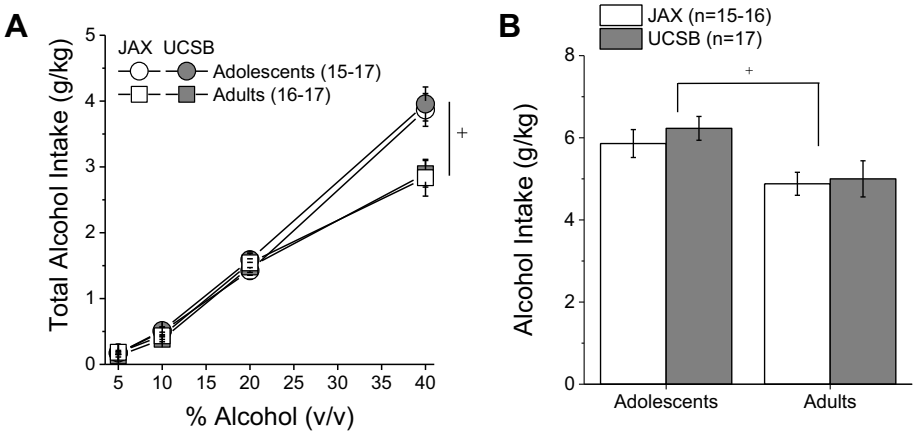

Fig. 5. Age-related differences in alcohol intake by female B6 mice under 4-BC procedures is not influenced significantly by the animal source. (*A*) A comparison of the dose-response function for alcohol intake exhibited by female adult and adolescent mice derived from The Jackson Laboratory (JAX) or bred in-house at UCSB (UCSB). (*B*) A comparison of average total alcohol intake exhibited by the different groups of female mice over the course of the 2-week drinking period. Data represent the means ± SEMs of the number of mice indicated in the figure. $^{+}$Denotes main Age effect ($p < 0.05$).

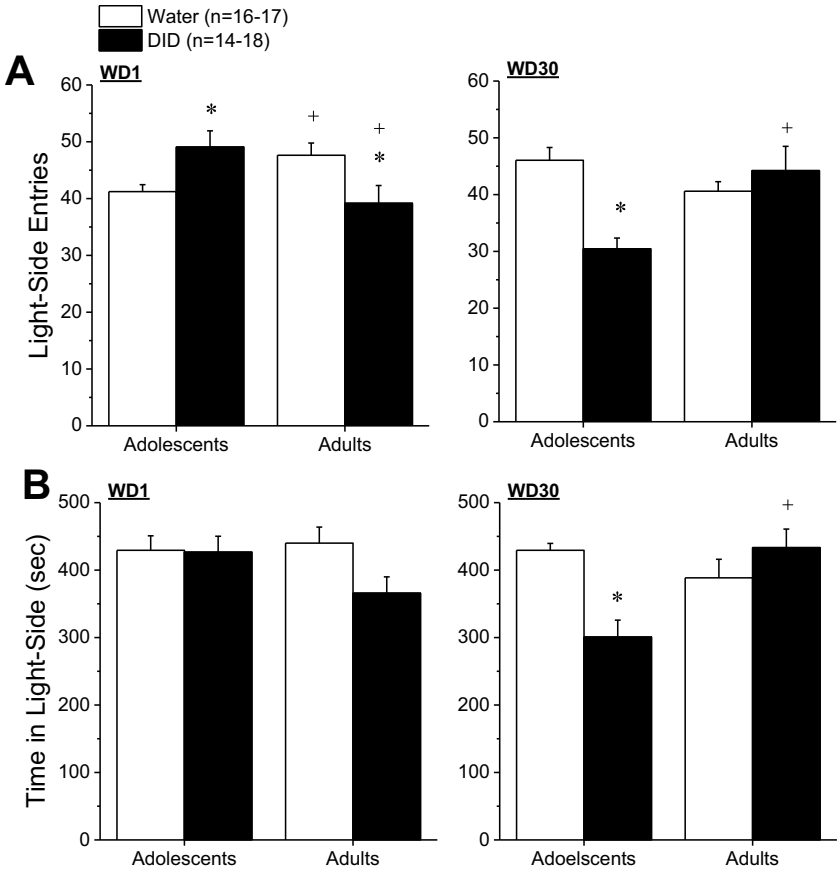

Fig. 6. Age-related differences in alcohol withdrawal-induced anxiety-like behavior exhibited by female B6 mice under light-dark shuttle-box procedures. (*A*) Depiction of the interactions between binge-drinking history, age of drinking-onset and withdrawal upon the number of light side entries, that reflected withdrawal-induced anxiety-like behavior in adult-onset binge-drinking (DID) mice on withdrawal day 1 (WD1) (*left*), and in adolescent-onset DID mice on WD30 (*right*). (*B*) Depiction of a similar 3-way interaction for the time spent in the light-side of the shuttle box. Data represent the means ± SEMs of the number of mice indicated in the figure. $^{*}p < 0.05$ vs. Water; $^{+}p < 0.05$ vs. Adolescents.

and alcohol-drinking adolescents [t(31) = 4.32, p < 0.0001]. In contrast, no alcohol effect observed in adolescent mice (*t*-test, p = 0.93), and no age-related difference in the latency to marble bury was observed in water-drinking controls (*t*-test, p = 0.53). On WD30 (Fig. 7A, right), we detected a main effect of binge-drinking only [DID effect: F(1,64) = 77.44, p < 0.0001; Age effect and interaction, p's > 0.50].

The pattern of group differences for the number of marbles buried was consistent with that observed for the latency to begin marble-burying (Fig. 7B) [DID × Age × Withdrawal: F(1,130) = 13.62, p < 0.0001], with no effect of animal source detected for this variable (see Supplemental Table 25 for complete results). Thus, the data were collapsed across the Source factor and the 3-way interaction deconstructed along the Withdrawal factor. On WD1 (Fig. 7B, left), we detected a significant DID × Age interaction [F(1,65) = 8.70, p = 0.004], that reflected more marbles buried by adult alcohol-drinking mice versus both their water controls [t(32) = 4.34, p < 0.0001], as well as alcohol-drinking adolescent mice [t(31) = 5.30, p < 0.0001]. No alcohol effect was detected in adolescent mice on WD1 (*t*-test, p = 0.91) and no age-related difference was observed between the water controls (*t*-test, p = 0.18). On WD30 (Fig. 7B, right), a significant Age × DID interaction was also detected [F(64) = 4.84, p = 0.03], with post-hoc comparisons indicating more marble-burying in both adult-onset [t(29) = 4.40, p < 0.0001] and adolescent-onset [t(32) = 6.90, p < 0.0001] alcohol-drinking mice relative to their respective water controls. The significant interaction reflected a modest, but significant, difference in the number of marbles buried between adult- and adolescent-onset water-drinking mice [t(31) = 2.07, p = 0.05], which was not apparent in their alcohol-drinking counterparts (*t*-test, p = 0.17).

Finally, the analysis of the time spent burying also indicated a significant DID × Age × Withdrawal interaction (Fig. 7C) [F(1,130) = 20.81, p < 0.0001]. As no Source effect or interactions were observed (p's > 0.30; see Supplemental Table 26 for complete results), the data were collapsed across Source prior to deconstruction of the significant interaction along the Withdrawal factor. On WD1 (Fig. 7C, left), we detected a significant DID × Age interaction [F(1,65) = 63.99, p < 0.0001], which reflected a longer burying time in alcohol-drinking adults, relative to their water-drinking controls [t(32) = 4.59, p < 0.0001], as well as adolescent alcohol-drinking animals [t(31) = 4.08, p < 0.0001]. No alcohol-related effect was observed in adolescent mice (*t*-test, p = 0.70). On WD30 (Fig. 7C, right), we detected a main effect of drinking [F(1,64) = 232.90, p < 0.0001], but no Age effect or interaction (p's > 0.90).

The present data from the marble-burying test provide novel evidence that certain signs of withdrawal-induced negative affect can persist into protracted withdrawal in female mice with a 2-week history of binge-drinking in adulthood. Further, the data from adolescent-onset alcohol-drinking females are consistent with those observed in males, indicating that signs of negative affect in this assay incubate during protracted withdrawal. Finally, the data fail to support any role for animal source in regulating behavior in this paradigm.

3.3.4. Partial replication of age- and withdrawal-related differences in alcohol withdrawal-induced negative affect in female B6 mice under forced swim procedures

Analysis of the latency to float during the forced swim test indicated a significant DID × Age × Withdrawal interaction (Fig. 8A) [F(1,130) = 7.53, p = 0.007], with no indication that animal source influenced this variable (see Supplemental Table 27 for complete results). Thus, the data were collapsed across Source to deconstruct the 3-way interaction along the Withdrawal factor. On WD1 (Fig. 8A, left), we detected a significant DID × Age interaction [F(1,65) = 34.55, p < 0.0001], which reflected longer latency to float in adult alcohol-drinking mice, relative to their water-drinking controls [t(32) = 7.31, p < 0.0001], but not relative to their alcohol-drinking adolescent

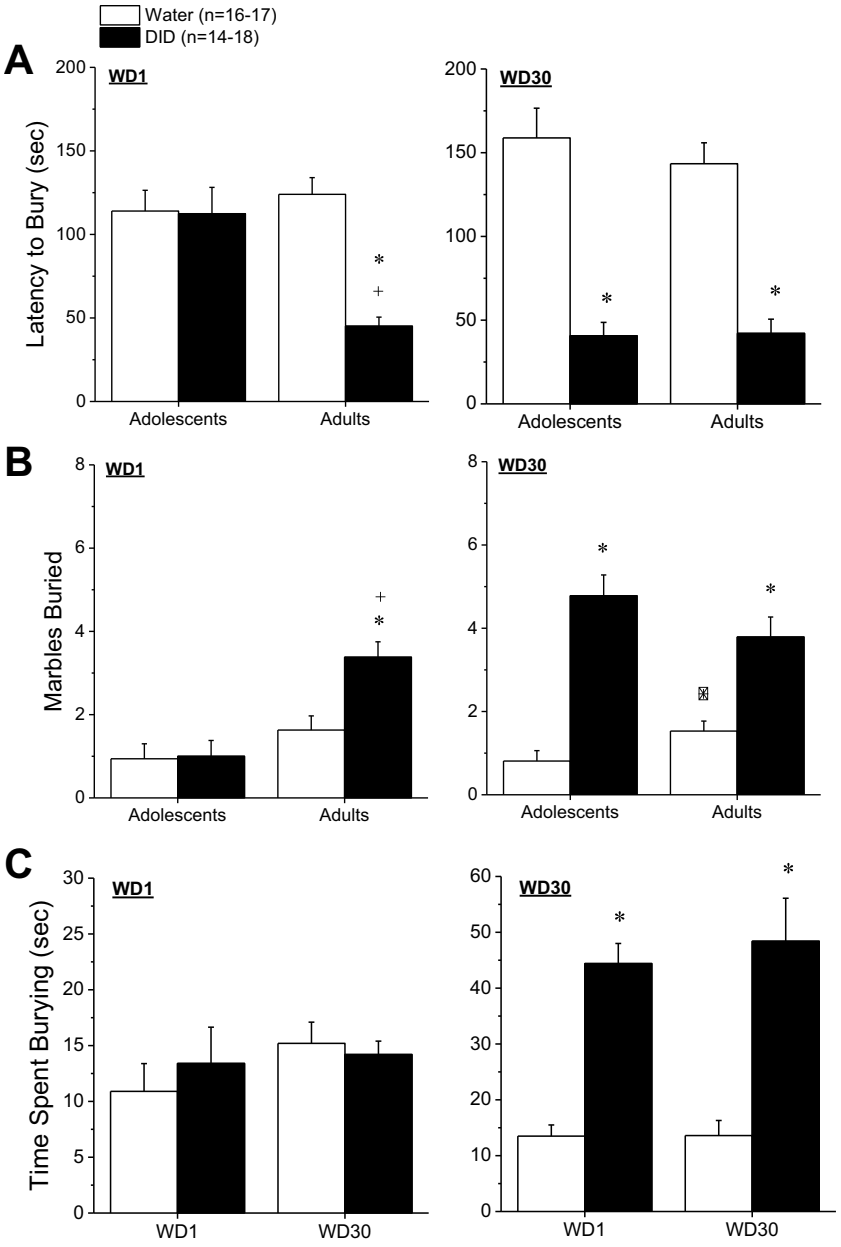

Fig. 7. Age-related differences in alcohol withdrawal-induced anxiety-like behavior exhibited by female B6 mice under marble-burying procedures. (*A*) Depiction of the interactions between binge-drinking history, age of drinking-onset and withdrawal upon the latency to marble-bury, that reflected withdrawal-induced anxiety-like behavior in adult-onset binge-drinking (DID) mice on withdrawal day 1 (WD1) (*left*), and in both alcohol-experienced groups on WD30 (*right*). (*B*) Depiction of a similar 3-way interaction for the number of marbles buried. (*C*) Depiction of the 3-way interaction for the time spent burying, which reflect no alcohol effect on WD1 (*left*), but more anxiety-like behavior in both adult- and adolescent-onset binge-drinking mice on WD30 (*right*). Data represent the means ± SEMs of the number of mice indicated in the figure. *p < 0.05 vs. Water; $^{+}$p < 0.05 vs. Adolescents.

counterparts (*t*-test, p = 0.21). No alcohol-related effect was observed in adolescent mice on WD1 (*t*-test, p = 0.98), although adolescent water-drinking mice exhibited a longer latency to float than did their adult counterparts [t(31) = 6.11, p < 0.0001]. On WD30 (Fig. 7A, right), we detected a significant effect of drinking [DID effect: F(1,64) = 127.98, p < 0.0001], a modest effect of Age [F(1,64) = 4.03, p = 0.05], but no significant interaction (p = 0.09). Irrespective of age of drinking-onset, alcohol-experienced mice exhibited a longer latency to float, relative to their water-drinking counterparts, with adult mice tending to have a longer float latency than the adolescent mice at this withdrawal time-point (Fig. 8A, right).

A similar pattern of group differences were observed for the time spent floating as that observed for the latency to float (Fig. 8B) [DID × Age × Withdrawal: F(1,130) = 8.74, p = 0.004], with no effect of animal source apparent from the results of the statistical analyses (see Supplemental Table 28 for complete details). As such, the data were collapsed across Source prior to deconstruction of the 3-way interaction along the Withdrawal factor. On WD1 (Fig. 8B, left), we detected a significant DID × Age interaction [F(1,65) = 15.07, p < 0.0001]. However, follow-up analyses indicated a shorter float time in both adult and adolescent alcohol-drinking females, relative to their water-drinking controls [for adults: t(32) = 3.12, p = 0.004; for adolescents: t(30) = 3.32, p = 0.002] and no age-related differences in float time observed in either water-drinking or alcohol-drinking mice (*t*-tests, for water: p = 0.90; for alcohol: p = 0.37). On WD30 (Fig. 8B, right), we detected a main DID effect only [F(1,64) = 26.99, p < 0.0001; other p's > 0.70], which reflected a shorter float time in alcohol-experienced animals, relative to their water-drinking controls.

In contrast to the above data, we detected no effect of alcohol upon the number of floats exhibited by either adolescent- or adult-onset drinking animals (no DID, Age or Withdrawal effects or interactions, all p's > 0.1; see Supplement Table 29 for complete results). Overall, JAX mice tended to exhibit more floats than UCSB mice [F(1,130) = 5.69, p = 0.02; JAX: 18.60 ± 0.78, n = 63; UCSB: 15.69 ± 0.96, n = 68], but source did not interact with any of the other independent variables examined (data not shown; see Supplemental Table 29).

The present data from the forced swim test provide novel evidence that a history of either adolescent- or adult-onset alcohol-drinking

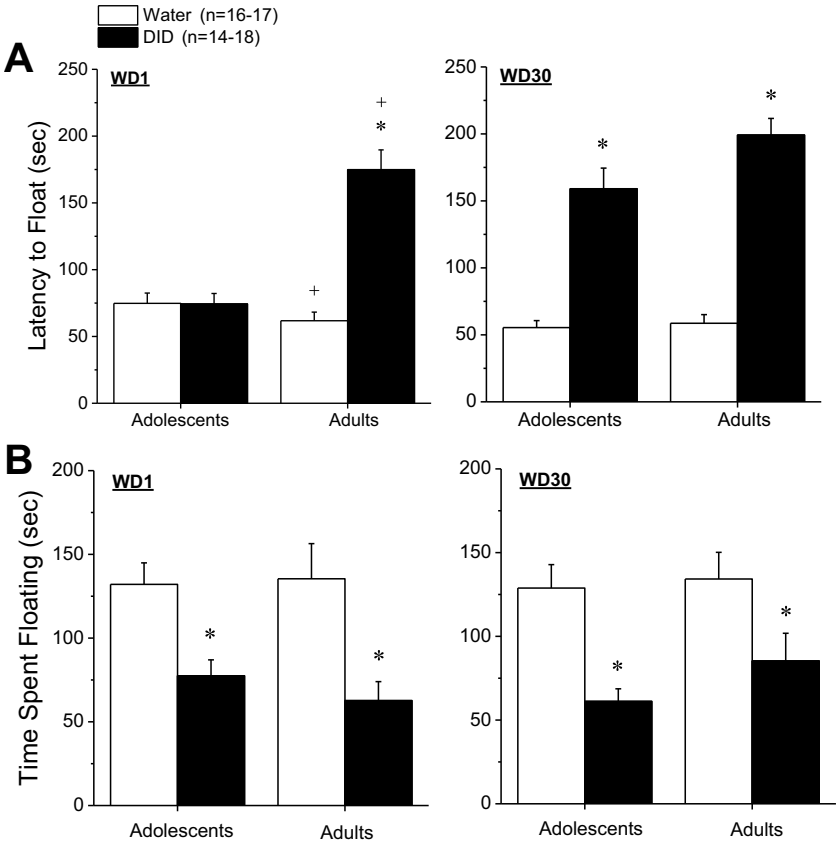

Fig. 8. Age-related differences in alcohol withdrawal-induced anxiety-like behavior exhibited by female B6 mice under forced swim procedures. (*A*) Depiction of the interaction between binge-drinking history, age of drinking-onset and withdrawal upon the latency to first float, that reflected withdrawal-induced anxiety-like behavior in adult-onset binge-drinking (DID) mice on withdrawal day 1 (WD1) (*left*), and in both alcohol-experienced groups on WD30 (*right*). (*B*) Depiction of the main effect of alcohol-drinking history upon the time spent floating. Data represent the means ± SEMs of the number of mice indicated in the figure. *p < 0.05 vs. respective Water; $^{+}$p < 0.05 vs. respective Adolescents.

elicits signs of withdrawal-induced anxiety-like behavior early in withdrawal in female mice that persist for at least 30 days. Interestingly, the direction of the effect of alcohol withdrawal upon floating behavior observed in the female mice with a history of adolescent-onset drinking is opposite that reported previously for males with a comparable drinking history (e.g., Lee et al., 2017b), suggesting potential sex differences in the manifestation of negative affect during alcohol withdrawal.

3.3.5. Replication of a sensitization of subsequent alcohol intake in female mice with a prior history of binge-drinking

Analysis of the average total alcohol intake exhibited by female mice during the week following behavioral testing indicated a significant Age × DID × Withdrawal interaction (Fig. 9) [F(1,130) = 5.87, p = 0.02], with no significant main effect of, or interactions with the Source factor (all p's > 0.10; see Supplemental Table 30 for complete results). Thus, the data were collapsed across the Source factor prior to deconstruction of the significant 3-way interaction along the Withdrawal factor. On WD1 (Fig. 9, left), mice with a prior binge-drinking history consumed more alcohol than prior naïve controls, irrespective of their age of drinking-onset [DID effect: F(1,65) = 35.10, p < 0.0001; no Age effect or interaction, p's > 0.30]. However, an analysis of drinking during protracted withdrawal indicated a significant DID × Age interaction [F(1,64) = 17.92, p < 0.0001], which reflected higher alcohol intake by adolescent-onset alcohol-drinking mice, relative to both their water-drinking controls [t(31) = 4.96, p < 0.0001] and adult-onset alcohol-drinking mice [t(30) = 4.53, p < 0.0001]. No alcohol-related effect was observed adult-onset alcohol-drinking females during protracted withdrawal (*t*-test, p = 0.93). These data indicate that, regardless of the age

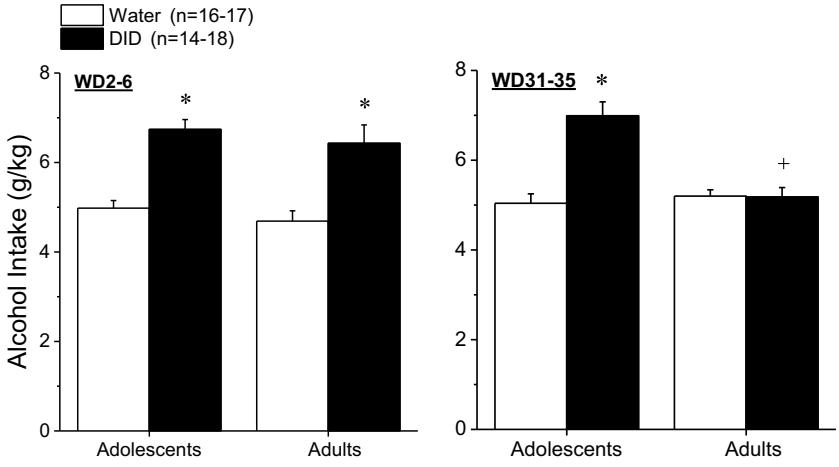

Fig. 9. Age-related differences in alcohol withdrawal-induced changes in alcohol intake exhibited by female B6 mice under 4-BC procedures. (*Left*) Depiction of the interaction between binge-drinking history, age of drinking-onset and withdrawal upon the average total alcohol intake during the latency to first float, that reflected withdrawal-induced anxiety-like behavior in adult-onset binge-drinking (DID) mice on withdrawal day 1 (WD1) (*left*), and in both alcohol-experienced groups on WD30 (*right*). (*Right*) Depiction of the main effect of alcohol-drinking history upon the time spent floating. Data represent the means ± SEMs of the number of mice indicated in the figure. *p < 0.05 vs. respective Water; $^{+}$p < 0.05 vs. respective Adolescents.

of drinking-onset, a prior history of binge-drinking promotes or sensitizes subsequent binge-drinking behavioral in early alcohol withdrawal in female mice. Further, these data indicate that this potentiating effect persists in female mice with a prior history of binge-drinking during adolescence.

4. Discussion

Binge-drinking is the most common form of alcohol abuse and alcoholism, particularly amongst adolescents and young adults (e.g., Chen et al., 2004; Harford et al., 2005; Martin and Winters, 1998; SAMHSA, 2010). In humans, early onset heavy drinking is a strong predictor of affective disorders in later life (e.g., Agosti, 2013; Grant et al., 2004; Kessler et al., 2005; Merikangas et al., 2007). Fitting with this, recent studies by our group have demonstrated using multi-bottle DID binge-drinking procedures that behavioral signs of negative affect (i.e., an anxious- and/or depressive-like state) incubate during protracted withdrawal in male B6 mice with a history of adolescent-onset binge-drinking (Lee et al., 2017b, 2018a, 2018b). However, clinical evidence indicates greater neuropsychiatric disturbances in female versus male adolescent-onset binge-drinkers (e.g., Hommer et al., 2001; Keller et al., 2010; Sharrett-Field et al., 2013; Schuckit et al., 1998). Thus, Experiment 1 was designed to extend our results from males to female subjects and test the hypothesis that alcohol withdrawal-induced negative affect would be more severe in female mice, particularly those with a history of binge-drinking during adolescence. To avoid interpretational confounds associated with age- and/or sex-related differences in reactivity to environmental stressors, Experiment 1 employed male and female mice bred in our UCSB colony.

4.1. "Sub-binge" levels of alcohol-drinking does not elicit a negative affective state during alcohol withdrawal

As detailed in Section 3.1, Experiment 1 successfully replicated published work indicating higher alcohol intake in female versus male B6 mice drinking under 2-h limited-access procedures (e.g., Melón et al., 2013; Metten et al., 2011; Rhodes et al., 2005; see Fig. 1). However, this initial experiment failed to replicate age-related differences in 2-h alcohol intake (e.g., Lee et al., 2016, 2017b, 2018a, 2018b; Melón et al., 2013; Metten et al., 2011). Further, the levels of alcohol intake, as well as BACs attained during drinking, were well below those expected of an animal model of voluntary binge-drinking (see Crabbe et al., 2011 for discussion). Not surprisingly, given their low alcohol intake, the mice in Experiment 1 exhibited no evidence of alcohol withdrawal-induced negative affect. This result fits with circumstantial evidence from our prior working suggesting that both the severity and longevity of the negative affective state observed during alcohol withdrawal with the amount of alcohol consumed. As mice were tested under ambient lighting over the course of 2 days, it is possible that circadian disruption may have mitigated our ability to detect water-alcohol differences in Experiment 1, particularly on the second day of testing. However, we have replicated water-alcohol differences in anxiety-like behavior expressed by male mice under both 1- and 2-day anxiety testing procedures. Thus, we attribute the failure to detect alcohol withdrawal-induced anxiety in Experiment 1 to the low level of alcohol intake.

Not expected, however, was the observation that mice with a history of repeated low-dose alcohol-drinking exhibited behaviors more consistent with a *hypo*-anxious state – a finding opposite that reported previously in B6 mice with a binge-drinking history. The "hypo-anxiety" exhibited by the mice in Experiment 1 was most notable in the light-dark shuttle box (Fig. 2C,D) and marble-burying assays (Fig. 2E–G), in which alcohol-drinking mice exhibited *less* defensive behaviors than their water-drinking counterparts. While we did not expect that alcohol withdrawal would impact every dependent variable assayed, we did expect, based on our prior work, to observe some age-related differences in the temporal manifestation of withdrawal-induced affective behavior across the different assays, with adult and adolescent mice exhibiting more pronounced behavioral effects, respectively, in early versus later alcohol withdrawal. Further, given that females consumed more alcohol than males during the 2-week drinking period, we anticipated sex-related differences in affective behavior that reflected the observed sex difference in alcohol-drinking. However, the behavioral changes observed in the alcohol-drinking mice of Experiment 1 did not vary systematically as a function of sex, age of binge-drinking onset or withdrawal, either within or between paradigms. We reasoned that the discrepancies in findings could reflect a number of procedural differences that we explored in follow-up experiments.

4.2. Habituation to the drinking environment appears to facilitate binge-drinking

Within the confines of this report, the results of Experiment 2 argue an important role for the duration of this acclimatization period for the manifestation of binge-drinking. When allowed a longer time to habituate to the drinking cages, the adult UCSB B6 mice in Experiment 2 consumed doses of alcohol at, or above, 4 g/kg in 2 h, which corresponds to BACs > 80 mg/dl (e.g., Lee et al., 2016, 2017b, 2018a). In fact, a comparison of the total alcohol intake of the adult female mice undergoing our 2-BC DID procedure in Experiment 1 versus 2 indicated an ~1 g/kg difference in intake between the two experiments (3.7 g/kg vs. 4.7 g/kg), with an even larger difference apparent in the adult males (2.7 g/kg vs. 4.4 g/kg). The alcohol intakes observed in Experiment 2 are more in line with those observed in our much earlier reports in which adult, male, B6 mice were continuously single-housed in their drinking cages (e.g., Cozzoli et al., 2014; Lee et al., 2016) than the results from Experiment 1. While requiring direct study, this collection of results, coupled with casual observations of high levels of exploratory behavior upon cage transfer (e.g., climbing on, or handing from, cage lid bars, hyper-locomotion and rearing; i.e., behaviors that are physically incompatible with drinking), argue that an acclimation period may be an important procedural factor when examining alcohol intake in a novel test cage.

As suggested from our earlier reports employing 3-BC versus 4-BC procedures, the alcohol intake of the UCSB B6 males in Experiment 2 was nearly identical under 2-, 3- or 4-BC procedures (Fig. 4C). Although total alcohol intake trended upwards as a function of the number of alcohol concentrations in the B6 females of Experiment 2 (Fig. 4C), the difference in intake across procedures was not statistically significant. The findings of Experiment 2 contrast with an earlier report indicating that alcohol intake by both male mice and rats increases proportionately with the number of bottles containing 10% alcohol presented under continuous-access procedures (Tordoff and Bachmanov, 2003), as well as our prior binge-drinking research using 1 vs. 4 concentrations (see Cozzoli et al., 2012). Whether or not our failure to detect a robust "bottle-dependent" increase in alcohol intake reflects the fact that we offered mice a choice of different alcohol concentrations ranging from 10 to 40% (v/v), the alcohol was presented under limited-access conditions and/or no water was available during the drinking period cannot be discerned from the design of the present studies. Nevertheless, from the results of Experiment 2, we conclude that, at least in adult B6 mice, no robust sex difference exists with the respect to the influence of the number of alcohol concentrations presented under limited-access upon the *manifestation* of binge-drinking, as operationally defined by NIAAA (NIAAA, 2007). Although, female B6 mice exhibit a tendency to consume higher amounts of alcohol when the range of alcohol concentrations available is restricted to higher concentrations, which might reflect a failure to titrate dosing. Such an interpretation would be consistent with clinical evidence that females tend to develop an AUD at a faster rate than males and exhibit greater AUD severity (e.g., Harford et al., 2005; Keyes et al., 2010; Schuckit et al., 1998). Whether or not such sex-related patterns in alcohol

consumption are apparent in other mouse strains or rodent species that exhibit lower alcohol intake than C57BL/6 mice requires further study.

4.3. Extension of the interaction between binge-drinking history, age of drinking-onset and withdrawal to female mice

As reported in male B6 mice from JAX, adolescent female B6 mice binge-drank more alcohol than their adult counterparts under 4-BC DID procedures in Experiment 3. While a prior alcohol-drinking study indicated that UCSB-bred male and female mice on a mixed C57BL/6J-129/SvlmJ background exhibit binge-like levels of alcohol intake under similar testing procedures as those employed herein (Quadir et al., 2017), we have never directly compared alcohol intake between commercially- versus in-sourced mice. The results of Experiment 3 indicted that the age-related difference in alcohol intake exhibited by female mice did not vary with animal source (Fig. 5), nor did animal source interact in any statistically reliable manner with the other independent variables with respect to either our behavioral indices of negative effect or subsequent binge-drinking. Based on these findings and our prior work with B6-hybrid mice (Quadir et al., 2017), we suggest that factors associated with animal transportation/relocation are not major determinants in the manifestation of binge-drinking or behavioral signs of negative affect following a 2-week period of binge-drinking, at least in female B6 mice tested within the confines of our laboratory setting. Future studies are necessary to determine whether or not the same is true for male B6 mice.

Male B6 mice from JAX with a 2-week history of binge-drinking exhibit age-related differences in the temporal manifestation of negative affect and excessive alcohol-drinking during withdrawal (Lee et al., 2016, 2017b). As reported for B6 males, the adult binge-drinking females from Experiment 3 manifested signs of anxiety-like behavior in the light-dark shuttle box, the marble-burying test and the forced swim test when assayed 24 h post-drinking. As also reported for males, the female binge-drinking adolescents exhibited no signs of anxiety-like behavior during early withdrawal in both the shuttle-box (Fig. 6) and marble-burying tests (Fig. 7). However, different from males, binge-drinking adolescent females spent less time floating than their water controls and the magnitude of this alcohol effect was comparable to that of the adult counterparts (Fig. 8). The observed alcohol effect on the float time of the adolescent binge-drinking females may simply be spurious, as these same mice exhibited no other behavioral signs of anxiety during early withdrawal. However, akin to the binge-drinking adults, the adolescent binge-drinking females also consumed more alcohol than their water-drinking controls during the days following the anxiety testing at the early withdrawal time-point (Fig. 9). These latter results suggest that adolescent females may be more prone than males to manifesting an anxiety-like state during early withdrawal from binge-drinking and, while relatively mild (i.e., only observed on one measure), the anxiety-like state may be sufficient to promote heavy drinking. As this finding is in line with human data indicated greater neuropsychiatric disturbances in female versus male adolescent binge-drinkers (e.g., Bekman et al., 2013; Squeglia et al., 2009), future work will involve a direct examination for sex differences in negative affect during early alcohol withdrawal and its relation to the propensity to consume the drug.

In our prior studies of males, no anxiety-like behavior is apparent during later withdrawal in adult-onset male mice with a 2-week history of binge-drinking (i.e., 30 days withdrawal), while their adolescent-onset counterparts exhibit very robust signs of anxiety-like behavior in the light-dark shuttle box and marble-burying tests, as well as signs of depressive-like behavior in the forced swim test and these signs of hyper-emotionality are associated with increased subsequent drinking. In Experiment 3, the pattern of age-related differences in the expression of anxiety-like behavior in the light-dark shuttle-box was very much in line with those observed previously in males; only adolescent-onset binge-drinking female mice exhibited signs of an anxiety-like state at 30 days withdrawal (Fig. 6). Similarly, only adolescent-onset binge-drinking females exhibited augmented alcohol intake when tested in protracted withdrawal (Fig. 9). However, on both the marble-burying and forced swim tests, both adult and adolescent females exhibited signs of anxiety-like behavior during protracted withdrawal; these effects were robust and observed across the majority of measures (Figs. 7, 8). Thus, a robust negative affective state (1) incubates during protracted withdrawal in female mice with a 2-week history of binge-drinking during adolescence and (2) persists in female mice with a 2-week history of binge-drinking during adulthood. We know from prior work that a persistent negative affective state can be observed in adult male B6 mice with a more extensive (30-day) binge-drinking history, which is correlated with cellular markers of hyperexcitability within the central nucleus of the amygdala and the bed nucleus of the stria terminalis (Lee et al., 2015). Thus, the possibility exists that the more enduring effect of binge-drinking exhibited by the adult B6 females in Experiment 3 merely reflects their propensity to consume more alcohol, resulting in more enduring neuroplasticity within the extended amygdala. Unfortunately, the single-sex approach of Experiment 3 precludes any firm conclusions in this regard. Nevertheless, the apparent sex-difference in the longevity of the negative affective state produced by a 2-week binge-drinking history in adult mice is very intriguing and warrants further, more directed, investigation.

4.4. Limitations of study

The purpose of this article was to report a failure by our laboratory to replicate a binge-drinking phenotype in C57BL/6 mice under specific limited-access drinking procedures and to summarize our efforts aimed at understanding the potential procedural bases of this failure. Given this, all data were obtained through studies of C57BL/6 mice and we cannot know if the findings generalize to other mouse strains or rodent species or to other drinking paradigms. Similarly, we cannot know if the age-related differences in alcohol withdrawal-induced anxiety exhibited by C57BL/6 mice in this or our prior work generalize across mouse strains or rodent species. While we have reported comparable withdrawal-induced anxiety in male C57BL/6 mice under both 1-day and 2-day behavioral test batteries, we cannot know how the magnitude of our group differences would compare if negative affect was assayed only in a single test or if mice were tested under low or red lighting conditions. Further, we have reported comparable alcohol intake and withdrawal-induced anxiety in male C57BL/6 mice housed singly or in groups; however, we have never systemically tested how individual versus group-housing impacts our dependent measures. Unless otherwise stipulated, the drinking and anxiety-testing procedures employed in the experiments described herein were conducted according to standard protocols that have been established in the laboratory for nearly 5 years. This *operando* facilitates data interpretation across studies and facilitates cross-study comparisons and the results of the present study were interpreted and discussed almost exclusively within the context of our own binge-drinking research. Indeed, the hypothesis that high levels of behavioral reactivity to the non-colony drinking cage interferes with focused drinking behavior under limited-access conditions was derived from behavioral observations during early pilot work when developing our “alternate cage” drinking procedures, and was supported by the differential findings from Experiment 1 versus 2. However, the reader is cautioned that our hypothesis was not tested in any parametric fashion and thus, the conclusions derived herein could benefit from additional follow-up study.

4.5. Conclusion

Herein, we show that a history of low-dose alcohol consumption is insufficient to elicit behavioral signs of negative affect in mice, irrespective of sex, the age of binge drinking-onset or the duration of alcohol withdrawal. We also suggest that the time allotted for group-

housed mice to habituate to a novel drinking cage is an important procedural variable regulating alcohol consumption in both male and female adult and adolescent mice and make recommendations that, when studying C57BL/6J mice, habituation periods of at least 45 min should be employed prior to alcohol presentation to minimize behavioral reactivity that is physically incompatible with drinking behavior. Further, we show that when allowed sufficient time to habituate to the drinking cage, both male and female mice will binge-drink alcohol when presented simultaneously with 20 and 40% alcohol over a 2-h period, but the level of consumption does not vary significantly with the opportunity to also consume lower alcohol concentrations. Finally, we show that prior binge-drinking history, age of drinking-onset and withdrawal are factors that can interact to influence negative affect in female mice, with evidence suggesting that the underlying processes and temporal patterning of emotional dysregulation may be different from that of males.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbb.2018.12.002.

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