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DIFFERENCES IN COMPULSIVE-LIKE ETHANOL CONSUMPTION IN
ADOLESCENT-TRAINED AND ADULT-TRAINED RATS EXPOSED TO ACUTE
ISOLATION STRESSORS

By

Maria J Lopez

Thesis Submitted in Partial Fulfillment of the Requirements for the Master of Science in
Experimental Psychology-Thesis with a Concentration in Behavioral Sciences

The Department of Psychology

Seton Hall University

August 2021

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We certify that we read this thesis written by **Maria J Lopez** in the Summer Semester 2021 and, in our opinion, it is sufficient in scientific scope and quality as a thesis for the degree of Master of Science.

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Abstract

The Pavlovian Conditioned Approach (PCA) procedure is an experimental procedure used with animals to induce compulsive-like behaviors directed towards an object conditioned stimulus (CS) paired with a sucrose pellet unconditioned stimulus (US) presented in a nearby food tray (sign tracking). When the object CS is an alcohol-containing bottle, the rats demonstrate compulsive-like interactions with the bottle object (e.g., approach, contact, and licking) that also results in alcohol consumption (drinking) in most rats. Compulsive alcohol drinking in a sign-tracking procedure is a result of the combined effects of schedule-induced compulsive-like behavior and the intake of alcohol (Tomie & Sharma, 2013).

The first goal of the present study was to investigate possible age differences in the acquisition of sign tracking of an alcohol bottle CS in Sprague-Dawley rats trained directly in their enriched home environment. If adolescent-trained rats are more susceptible to the compulsive-inducing effects of the sign-tracking procedure or to the pharmacological effects of alcohol, then adolescents should show more sign tracking than the adults. However, the results showed faster acquisition and greater asymptotic sign tracking of an alcohol bottle in the adult-trained rats. The second goal of the study was to investigate the effects of an acute stressor on asymptotic sign-tracking performance in both groups once the adolescent rats reached an adult age. I tested the possibility that rats exposed to acute stress conditions in adulthood are more likely to show stress-induced increases in compulsive-like behavior if the compulsive behavior was learned during adolescence rather than during adulthood. All rats were subjected to acute isolation stress by placing them in restrictive home cages (i.e., isolated from their home environment). Results showed that overall, the rats who were trained in the PCA procedure during adolescence did not display increased compulsive-like behaviors in adulthood during

acute stress conditions compared to rats who were initially trained in adulthood; the adolescent-trained rats exhibited less sign tracking than the adult-trained rats. Stress-induced changes in compulsive-like behaviors were seen in adult-trained rats; the adult-trained rats exhibited increased sign tracking. Age differences in goal-directed behaviors, goal tracking, were also seen. Overall, the results demonstrate that compulsive-like alcohol consumption is readily evoked by the sign tracking procedure in adolescent and adult rats even when trained in an enriched home environment, providing an opportunity to examine the effects of age and environmental factors on the acquisition and modulation of compulsive-like behavior.

Introduction

Alcohol is a legal substance readily available for sale to anyone over the age of 20 in the US but underage use is prevalent. According to the most recent National Survey on Drug Use and Health (SAMHSA, 2019), 51.1 percent of people aged 12 or older reported current alcohol use, of which 48.0 percent were binge users. A brain disease view of alcohol addiction emphasizes evidence of brain changes resulting from excessive alcohol use. With repeated exposure to alcohol, the brain begins to adapt to compensate for the neurochemical changes needed to function normally (Becker, 2008). Interestingly though, only 5.4 percent of the people aged 12 or older who were current alcohol users met criteria for an alcohol use disorder diagnosis which included abuse and dependence. Therefore, there is a large proportion of alcohol users that do not develop an addiction even after excessive use as the brain disease model would dictate.

Prevalence of adolescent alcohol use. The prevalence of alcohol use in adolescence varies from that of young adulthood and adulthood. Approximately 55 percent of adults aged 26 and over reported current alcohol use (SAMHSA, 2019), with young adults aged 18 to 25 mirroring that percentage. However, only 9 percent of adolescents aged 12 to 17 reported current alcohol use. Even though there is a low prevalence in adolescent alcohol use, the transition in use from adolescence to adulthood is unknown and likely to be a result of a complex interaction involving environmental experiences, behavioral predispositions and maturational processes, thus alcohol use during a critical period of neurobiological development is concerning. The brain undergoes extensive maturation during adolescence and early adulthood (Griffin, 2017; Spear, 2013). Cognitive processing becomes more efficient as connections with multiple brain regions are increased and strengthened. Axons become more insulated with myelin, dendrites experience

branch growth, and synapses used often are strengthened and those used less are diminished, making communication in the brain more effective. Specific brain regions also experience maturation. Specifically, one of the brain areas directly involved with controlling impulsivity, the prefrontal cortex, is still developing. The prefrontal cortex integrates environmental cues for decision making while regulating emotions and behaviors. The prefrontal dopamine system assists in this task through its inhibitory effect, allowing adolescents and young adults to delay impulsive behaviors. The environment also plays a critical role during adolescent and young adulthood neurodevelopment (Griffin, 2017). Previous experiences determine which synapses become heavily used, which are strengthened, and those that are no longer useful are abandoned. This enables cognitive processing to become quicker but also eliminates the full range of options in which to adapt to environmental cues. Stronger synapses facilitate the utilized reactions. Environmental changes are also prevalent in adolescence and young adulthood with high school, college, and post-college transitions. Therefore, the strengthened synapses learned in the critical periods of adolescence and young adulthood are extremely important when discussing substance use and its early onset exposure (National Institute of Drug Abuse, 2014). The medical model of addiction suggests that continuous drug use will disrupt normal brain development in adolescents and pathologically alter brain structures in adults and that these changes may still be present long after the drug use has ceased.

Vulnerability to alcohol use among adolescence. Many studies suggest that adolescents are more vulnerable to alcohol abuse than adults (Brown & Tapert, 2004; Crews et al., 2004), citing physiological evidence regarding adolescent sensitivity to the effects of alcohol. Moreover, adolescents who repeatedly use alcohol are more likely to continue use and become addicted to it later as adults (McCambridge et al., 2011; Merline et al., 2008). Chronic and heavy

adolescent alcohol use were determinants for the same pattern of use in adulthood and were associated with both alcohol abuse and dependence in adulthood. Although there is substantial evidence that there can be a genetic component to alcohol abuse and addiction there is no one “alcoholism gene”, but many genes that likely play a role. For example, some genes related to alcohol metabolism (ADH1B and ALDH2) are strongly correlated with alcoholism dependence’ (Edenberg & Foroud, 2013); however, the consensus in the medical and psychology fields is that environmental and social components also heavily affect susceptibility. One possibility for the transition from adolescent to adult alcohol use is the increased attribution of incentive salience to cues associated with alcohol and alcohol use.

The Incentive Sensitization Theory of addiction, proposed by Robinson and Berridge (1993), is a biopsychosocial theory of addiction that explains the difference between the ‘liking’ (pleasurable effects) of a drug and that of its ‘wanting’ (incentive salience) in an attempt to catalogue how addiction cravings (i.e., compulsive wanting) are formed, persist, and are independent of positive and negative reinforcement. Although wanting and liking appear to be complementary motivational processes subjectively, research indicates that in addiction they are dissociated such that the wanting of a drug is much greater than the pleasurable experience of the drug (Robinson & Berridge, 2008). Drug-paired cues themselves gain incentive value through a Pavlovian conditioning process. The cue, now imbued with incentive salience, triggers wanting that elicits a peak of temptation to seek the drug-paired cue. When the cue is located and approached the opportunity is provided to self-administer the drug itself, even when the drug is no longer as effective (not as ‘liked’) as it once was. Sensitization, which refers to an increased response to a stimulus caused by repeated presentation of the stimulus alone, is a well-known phenomenon in the learning and behavioral pharmacology literature and is most often

investigated with drugs (e.g., amphetamine) that induce locomotor activity. For example, when a fixed dose of amphetamine is administered repeatedly the locomotor activity induced by the drug increases with each presentation. Non-drug incentive stimuli may also show a sensitization effect with repeated stimulus exposure, a phenomenon that is opposite of stimulus habituation. The incentive sensitization theory posits that in addiction the normal process of attaching incentive salience to a repeatedly presented drug-paired cue becomes pathologically “hypersensitized”. Robinson and Berridge (1993, 2008) argue that this incentive sensitization process results in the compulsive wanting of a drug and its associated cues. One such experimental procedure that induces incentive learning in rodents that resembles compulsive-like wanting is the Pavlovian Conditioned Approach (PCA) procedure.

During the PCA procedure, the repeated presentation of an object conditioned stimulus (CS) is immediately paired with the response-independent delivery of a rewarding unconditioned stimulus (US). After repeated object CS – food US pairings in the PCA procedure, one of two dominant behaviors may develop: goal tracking or sign tracking. Subjects that utilize the appearance of the lever CS as a mere signal to the impending US delivery interact with the US location displaying anticipatory goal-tracking behavior. Subjects that approach and interact with the CS itself, are displaying sign-tracking behavior. Sign-tracking behavior emerges in these animals even though behavior directed towards the CS does not affect the delivery of the US. A brief lever-insertion is a popular CS in rat studies because rats can interact with the lever by sniffing, touching, biting, and placing downward pressure on the lever allowing for the latter behavior (i.e., lever pressing) to be automatically recorded. Other studies utilize a bottle as the object CS and interactions with the spout (sniffing, touching, biting, and licking) are recorded as contacts. More importantly, when rats lick the bottle, they also consume its content. Tomie and

colleagues have argued that the PCA procedure with an alcohol bottle CS effectively models compulsive alcohol use and addiction (Tomie & Sharma, 2013). It is important to note that when the bottle CS is filled with water, sign-tracking behaviors emerge towards the bottle as well as compulsive water consumption. This behavior is compulsive because the rats are not deprived of water, that is thirst is not driving the drinking behavior. When the bottle CS is filled with alcohol, compulsive alcohol consumption behaviors are seen at higher levels than the water-filled bottle CS, indicating that sign tracking of a bottle is driven primarily by incentive value attached to the bottle object itself plus pharmacological (or caloric) effects when the bottle content is alcohol (for review, see Tomie et al., 2008).

Interestingly, many studies with the PCA procedure using lever CSs demonstrate that there are substantial individual differences in the responses to the object CS (Flagel et al., 2007). During the CS presentation, goal trackers investigate the location where the food is delivered by head poking repeatedly into the food tray in anticipation of the food delivery. These animals, therefore, are not displaying compulsive-like behavior directed towards the lever CS and are considered to lack the “addiction vulnerability” phenotype. In contrast, sign tracking is described as compulsive-like behavior because it is non-productive and excessive which suggests an “addiction vulnerability” phenotype. Thus, sign-tracking behavior is considered an indicator of excessive incentive value placed on the CS by virtue of being paired with a US in accordance with the incentive sensitization theory.

These studies looking at individual differences in sign tracking and goal tracking have suggested that most rats are either sign trackers or goal trackers, with the former being more vulnerable to assigning incentive value to reward-paired cues and therefore when the reward is a drug or alcohol, they are more vulnerable to addiction (Saunders & Robinson, 2011). However,

more recent studies suggest that this sign- versus goal-tracking dichotomy was overstated. Most rats engage in both behaviors and rats showing predominantly goal tracking early in training can gradually shift toward sign tracking with more experience with the procedure (Srey et al., 2015). Moreover, the initial belief that only sign trackers were more vulnerable to addiction-like behaviors (e.g., drug self-administration) appears to have been overstated since even some rats categorized as goal trackers can also develop addiction-like behaviors given prolonged exposure to drugs and alcohol (Colaizzi et al., 2020). In contrast to studies using a lever CS, most studies using an alcohol bottle CS have not made this sign tracking versus goal tracking distinction, focusing instead on the measure of sign tracking since all rats show at least some sign tracking performance (Tomie et al., 2002; Tomie et al., 2005). The present study utilized an alcohol bottle for the CS to induce compulsive-like alcohol consumption in adolescent and adult rats. Unlike most prior bottle-CS PCA studies goal tracking was also measured.

Adolescent rats' sign tracking performance. Researchers have hypothesized that adolescent rats are more vulnerable to attribute incentive value to reward-paired cues than adults and therefore may more readily respond to drug or alcohol associated cues. If adolescents more easily attribute incentive value to reward-associated cues, they would be expected to show greater sign tracking than adults. Surprisingly, several studies indicate that adolescent rats show less sign tracking than adult rats (Anderson & Spear, 2011; Doremus-Fitzwater et al., 2010). However, rats who were exposed to sign-tracking procedures in adolescence displayed increased sign-tracking behaviors later as adults compared to adult rats who did not have the early onset exposure (Anderson & Spear, 2011; Tomie et al., 2005). Anderson and Spear (2011) also investigated the relationship between voluntary alcohol consumption outside of the sign tracking procedure and sign tracking of a lever CS. They found a positive relationship between sign

tracking and alcohol consumption only in adult rats that experienced sign tracking in adolescence. McClory and Spear (2014) found that alcohol exposure in adolescence increased sign-tracking of a lever CS in adulthood but not when initial alcohol exposure occurred in adulthood. These results suggest that earlier experiences, including the tendency to assign incentive value to reward-paired cues, manifest behaviors that emerge later in adulthood and experience with alcohol in adolescence may enhance these effects. In these studies, alcohol was given independently of sign tracking. In the present study the use of an alcohol bottle CS rather than a lever CS allows the rats to experience alcohol consumption concurrently with sign tracking. Thus, this procedure provides better face validity for sign tracking as an animal model of compulsive-like alcohol consumption that may ultimately result in alcohol abuse or addiction. Moreover, to improve on the face validity of the sign-tracking model, the rats were tested directly in their home environment (rather than in isolated test chambers) consisting of a large cage tower with multiple cage mates and various novelty objects to engage with for enrichment.

The current study also conducted two- bottle preference tests to measure the ‘liking’ of the ethanol solution which the Incentive Sensitization Theory differentiates from the compulsive ‘wanting’. The preference of a particular substance given another option, in this case water, answers the question of whether there is a liking of ethanol itself regardless of a wanting to consume it which is measured through the PCA procedure. Studies (Tomie et al., 2004) have gathered supporting evidence that these two measures are not linked together, that is increased sign tracking of alcohol does not transfer to a preference for alcohol outside of the PCA procedure. Therefore, we posited that increased sign tracking – that is, ‘wanting’ - would not equate to an increase in preference – that is, ‘liking’ - as measured in the preference tests outside of the home environment. Finally, the use of an alcohol bottle CS in the home environment

provided the opportunity of investigating how stressors may impact on the compulsive-like behavior (sign tracking) and purposeful goal-directed behavior (goal tracking) of rats while in their home environment.

Vulnerability to stress. Stressors are a normal occurrence in life regardless of developmental stage. Adolescents and young adults are exposed to multiple waves of stressors with transitions to high school, college, and post-college. While all developmental stages encounter stress, adolescents typically react to them physiologically different than adults do. Adolescents have a heightened response to stress compared to adults. When a stressor occurs, regardless of it being physical or psychological, one of the brain responses for regulation is for the hypothalamic-pituitary-adrenal (HPA) axis to mediate the secretion of corticotropin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), and cortisol. Whereas a mature brain will respond to a stressor by secreting ACTH and cortisol at standard levels, the frequency and duration of secretion is increased in adolescents (Romeo, 2013). The shift from adolescent to mature stress responsiveness occurs in stages, with each hormone normalizing at different times until reaching adulthood levels. Late adolescence marks the highest cortisol secretion levels from a stressor compared to early adolescence (Romeo, 2013). In animal studies, stress vulnerability has been found in adolescence, specifically late adolescence, mirroring the effects found in human studies (Jankord et al., 2011). The brain areas that assist in terminating the hormonal stress response are undergoing maturation during adolescence and into young adulthood, possibly rendering them sensitive and highly responsive. Due to these hormonal shifts and continuous brain maturation, adolescents may be vulnerable to stressors especially while they are undergoing their transition into adulthood. However, the effects of these stressors may linger into adulthood such as previously noted sign tracking age effects.

Social isolation is an experimental stressor protocol utilized in animal studies to evoke developmental influences in a natural, species-typical manner (Heinrichs & Koob, 2005). Anderson, Bush, and Spear (2013) initially looked at age differences in rats reared in pairs and found that adult rats displayed more sign tracking than adolescents. However, when an 11-day period of isolated housing was implemented in a subsequent experiment the age difference was no longer observed. There was no noticeable sign-tracking behavior for either group. They did not compare the sign tracking measures caused by the isolation stressor to the age groups' previous baseline measures. Therefore, whether sign tracking decreased in adult rats or if sign tracking increased in the adolescent group due to this stressor is unknown. The same study then implemented the isolated housing condition along with food deprivation in another set of adolescent and adult rats. They found that this set of stress conditions increased sign-tracking behaviors in both age groups when compared to the original pair-housed baseline measures. They analyzed this comparison further and found that this stressor effect was substantially greater in the adolescent rats than the adult rats. A similar increase in sign tracking induced by social isolation has been reported elsewhere (Beckman & Bardo, 2012). The authors interpreted the greater change in sign tracking in isolated than pair-housed adolescent and adult rats as a stress effect and suggested that paired housing may serve as a social buffer against the stressful effects of isolation. The animals in the current study were housed in groups of four in an environment typically described as "enriched". The use of enriched environment rearing provided an opportunity to examine the effects of acute social isolation stress during PCA and preference tests by temporarily moving the rats from their enriched home environment to an isolated cage. Interestingly, a recent cross-sectional study with adults reported an increase in alcohol consumption associated with quarantine isolation during the COVID-19 Pandemic

(Grossman, Benjamin-Neelon & Sonnenschein, 2020). I was interested in determining if acute isolation stressor conditions differentially alter performance of compulsive-like behavior (sign tracking of an alcohol bottle) and purposeful goal-directed behavior (goal tracking).

Summary of Current Study

The purpose of the current study was twofold. The first was to determine if the reported age differences (adolescents versus adults) in the acquisition of incentive learning, induced by the PCA procedure with a lever CS, is also observed with an alcohol bottle CS directly in the home environment by including the sign-tracking apparatus as a part of the home cage. A PCA procedure directly in an “enriched” home environment is a novel approach that has not been previously done with the PCA procedure. These procedural differences were chosen to improve on the face validity of sign tracking as an animal model of compulsive-like alcohol use in humans. Adolescent and adult rats were trained in a total of 18 sessions of the PCA procedure and both sign-tracking and goal-tracking behaviors were measured. Secondly, the current study also examined how acute isolation stressors affect fully established sign-tracking and goal-tracking behaviors. Studies of stress manipulations vary substantially in the animal literature and consist of a wide range of stressor types (e.g., pharmacological, natural) that are administered in different durations (acute, chronic) and in different temporal relationship to the main dependent measure of interest. The results of these studies also vary substantially depending on the parameters of the study, the characteristics of research subject (species, strain, sex, age) and how the stress is operationally defined (specific experiences, behavioral changes, or physiological measures) and many other factors (see Patchev & Patchev, 2006; Scharf & Schmidt, 2012). In the present study I was interested in potential changes in compulsive-like (sign tracking) and goal-directed action (goal tracking) during the experience of a natural and short-term stressor.

Because the rats were housed in an enriched social environment, I had the opportunity to introduce a natural stressor. Studies show that acute social isolation disrupts several measures of motivated behavior (Lerser & Wagner, 2015; Panksepp & Beaty, 1980). I assumed that isolation in a restricted cage would be stressful to rats accustomed to living in an enriched home environment and therefore was interested in examining performance during the ‘unnatural’ experience of isolation from the home environment and after a return to the ‘natural’ home environment. The asymptotic performance at the end of the acquisition phase became the baselines for each group which were then compared to the same measures following the stressor manipulations. This comparison was not completely addressed in the Anderson, Bush, and Spear (2013) study. The adolescent rats began PCA training in adolescence, but they were adults when two isolation stressor conditions were introduced: ‘environmental’ (removed from the home enriched environment and isolated with a cage mate) and ‘social’ (removed from the home enriched environment and isolated alone). While the ‘social’ stressor condition also includes an environmental stressor by removing the rats from their home towers, the label will be used to discriminate between the two stressors. The adults were trained in the PCA procedure as adults and experienced the isolation stressors as adults. If the effects of adolescent exposure to alcohol use emerge later in adulthood (Anderson & Spear, 2011; Tomie et al., 2005), it would be beneficial to know if common experiences such as acute stress affects these adolescent-learned behaviors more than adults first exposed to alcohol and PCA training in adulthood.

Although prior published studies have not utilized enriched environment housing as the standard rearing procedure and have not examined stressor effects on already fully established sign-tracking behavior, prior results have suggested that social stressors increase sign-tracking behavior. Therefore, I posited that the isolation stressors might increase sign-tracking behavior

for the adult rats exposed to PCA earlier in adolescence, hereinafter referred to as adolescent-trained, relative to the adult rats exposed to PCA in adulthood, hereinafter referred to as adult-trained.

Method

Subjects

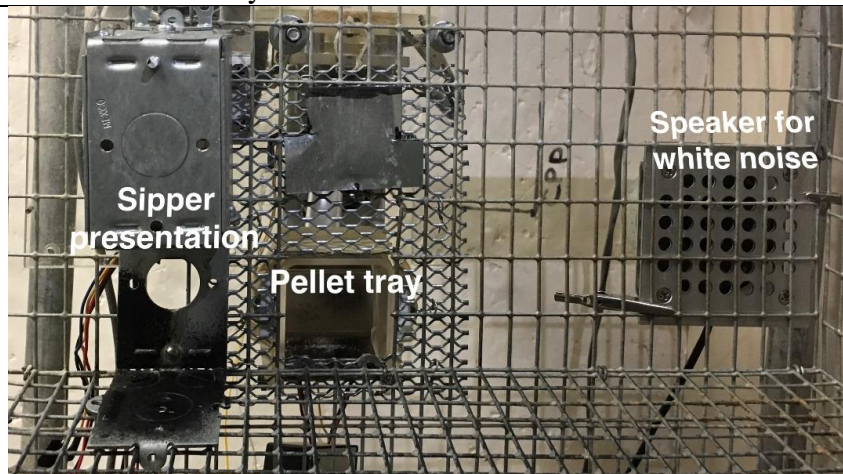
A total of sixteen male Sprague-Dawley rats were purchased from Envigo (Indianapolis, IN) at 21 days of age. Each animal was randomized into one of two groups upon arrival. Eight of the animals were randomly assigned to the adolescent-trained group and placed in groups of four to one of two towers, for a total of four rats per tower. The remaining eight animals were assigned to the adult-trained group and placed in groups of four to one of two towers, for a total of four rats per tower. All animals were provided with food (Teklan Rodent Diet, 7102) and water *ad libitum* throughout the entire experiment. All animals were maintained on a 12-hour light-dark cycle, with the light turning on at 8am. IACUC approval was obtained prior to data collection.

Materials

Four towers made of wire-mesh were used in this study (Martin Cages, Inc, Nanticoke, PA, Model # H-600HR; see Figure 1). The towers' dimensions were 18" W x 11" D x 24" H. Each tower sat atop a deep plastic pan lined with soft corn cob bedding (Teklan 7087c) which was considered the bottom floor and held the food trays for *ad libitum* access. The towers also had a lower landing, center floor, and an upper landing. Two water bottles with attached sippers were provided on the lower landings for *ad libitum* access. Because the animals were tested in their home cages in the colony room, a black cloth curtain covered the front of each tower to minimize visual distraction from any activity in the colony room. When servicing the animals, the curtain was withdrawn to the top of the rack holding the cage towers. Novel objects such as chew toys and paper towel rolls were scattered in each tower and rotated weekly. A PVC pipe (10.2 Dia x 30.5 L cm) was provided to each tower for hiding on the center floors as well as



Lopez, M. J. (Photographer). (2019). *Enriched environment towers* [photograph]. South Orange, NJ: Seton Hall University.



Pra Sisto, A. J. (Photographer). (2019). *Sign tracking apparatus, located on top landing of enrichment towers* [photograph]. South Orange, NJ: Seton Hall University.

Figure 1. An example tower used in the experiment. The top figure shows the full tower housing 4 rats. The bottom floor was a plastic tray covered with corn cob bedding. Food hoppers were on the left wall and a wood nest box was placed on the right side. A PVC pipe was another hiding area and placed on the middle floor. Between the middle and bottom floor was a platform on the right where the rats could access 2 water bottles. Above the middle floor on the left was the sign tracking platform where animals were trained in the sign-tracking procedure. The lower figure shows the location of the items on the sign tracking platform that were activated when a rat was restricted to this platform for sign tracking- sipper access hole (bottle CS), pellet tray (food US) and a speaker (for white noise).

wooden nesting boxes (30.5 W x 20.3D x 12.7H cm) on the bottom floor. The listed objects were utilized to create the enriched housing environment.

Sign-tracking chambers were created on the upper landings of each tower. The upper landings were enclosed with a clear partition during PCA adaptation and procedures so that the animals remained on the upper landing for the duration of training. A retractable bottle and sipper tube device (Med Associates Inc., St. Albans, Vt, ENV-252M) was mounted on the left-hand side of the upper landing. The sipper tube's tip was flush with the wire-mesh wall when presented as the CS during PCA procedures that allowed contact. A lickometer controller (Med Associates Inc., ENV-250B) recorded these contacts/licks on the sipper until it was retracted at the end of each sign-tracking trial. A pellet dispenser (Ralph Gerbrands Co) filled with sugar pellets (Noyes, 45 mg) was located on the right side of the retractable bottle and sipper tube device, also flush against the wire-mesh wall. A photobeam detector (Med Associates Inc., ENV-303HD) recorded head poking into the pellet dispenser. A speaker was located on the right-hand corner of the upper landing and provided background white noise throughout PCA procedures. All equipment was controlled by Med Associates interface equipment and programs written in Med PC (Med Associates Inc., St. Albans, Vt).

Individual stainless-steel cages (17.8 W x 24.4 L x 17.9 H cm) were used for the short-term preference tests. These cages were mounted in a row on a wooden frame in a different testing room. Standard shoe boxed-sized plastic cages with a 141 square inch floor were utilized for the social isolation stressors and to hold the remaining animals during PCA procedures. During social isolation stressor manipulation black contact paper was wrapped around the outside of the cage to prevent visual access to neighboring cages and the activities in the colony

room. Ethanol (EtOH) solutions were prepared by diluting 180-200 proof EtOH with tap water to the appropriate concentrations for the ethanol exposure, PCA procedures, and preference tests.

Procedure

A summary of the procedure timeline is shown in Table 1. All rats were housed in the enriched tower cages through the duration of the experiment, therefore the procedures described for each week in the table also included enrichment, but the text is omitted because of space limitations. The adolescent- and adult-trained groups were handled in the same way in terms of general animal husbandry and enrichment protocol (e.g., providing novel objects weekly). They were also treated similarly with respect to ethanol pre-exposure, PCA training and preference tests, and stressor manipulations except as noted below at different ages. Adolescence and adulthood were defined using widely accepted ages for laboratory rats (Spear, 2000) of postnatal day (P) 28 and 70, respectively.

Phase 1: Acquisition

Ethanol pre-exposure. The adolescent-trained group began initial ethanol exposure on P35 and the adult-trained group on P98 to adapt them to the ethanol availability and encourage ethanol consumption. One of the water bottles in each home tower was replaced with an ethanol solution. The ethanol solution began at a 2% concentration and systematically increased in increments of 1% up to 5%. The adult-trained group was given one less week of pre-exposure because they promptly consumed the ethanol more so than the adolescent-trained group. It was not possible to determine which animal was drinking the ethanol in the home cage because of the social housing. Thus, to determine a rat's willingness to consume ethanol each animal received a 20-minute two bottle choice test weekly in a separate testing room. During the choice test, each animal was provided a bottle containing water along with a bottle containing the latest ethanol

concentration. The left-right bottle placements were reversed for each test to prevent place preference. Intakes were evaluated as a preference for ethanol over water and as the absolute amount of ethanol consumed.

PCA training procedure. All animals received two days of adaptation to the sign tracking landing. During adaptation the rats were confined to the top landing of the home tower by installing a plexiglass barrier and sugar pellets used as the US during training were placed in the food tray for the rats to discover and consume. After adaptation was completed, the PCA procedures commenced. The adolescent-trained group began PCA exposure at P56 and the adult-trained group on P112. During PCA procedures, all animals were removed from their towers and placed in a standard cage with their cage mates in another room. The clear partitions were placed in each upper landing as in adaptation. The bottle CS was filled with a 5% ethanol solution and weighed. The animal being tested was also weighed before each PCA session and then placed into their home tower's sign tracking landing. The black curtain was then lowered from the top of the cage rack holding the towers. The white noise was turned on to indicate a PCA session began. Each session consisted of 25 trials where the bottle CS was presented for 10 seconds followed by a sugar pellet dispensed into the holder. The 25 trials were separated with a 60-second inter-trial interval. After each PCA session, the bottle CS was weighed again and recorded prior to beginning another animal's session. After the scheduled day's sessions were complete, the clear partitions were removed and the animals placed back into their respective towers. During this phase 5% ethanol and water was placed on the towers over the weekend to maintain an opportunity for rats to consume ethanol. Four two-bottle preference tests were conducted throughout PCA exposure during weeks 1, 2, and 5 of training sessions to monitor individual rats' preference (or 'liking') for the ethanol.

Phase 2: Acute Social Isolation Stressors. Following PCA acquisition, the social isolation stressors were conducted. The adolescent-trained group began the stressor conditions on P105 and the adult-trained group on P154. The animals were placed in standard cages under two conditions, either alone (social) or with a cage mate (environmental). Two animals in each tower were randomly assigned to either condition to counterbalance the order. The standard cages were wrapped in black contact paper to block sight of the external environment. Food and water were provided *ad libitum*. After a total of three days in the first isolated stressor condition, the animals were returned to their home towers for three days of recovery. The animals then received the second stressor condition for three days, followed again by three days of recovery in their home towers. A PCA session and preference test were conducted every 24 hours for the entire duration of Phase 2. For the PCA tests during the stressor condition the rats were returned briefly to their home cage landing for a testing session (the same procedure as the training sessions) and at the end of testing they were immediately returned to the isolated cage. During the recovery days the rats were again living in their home towers therefore after a test session they remained in their home cage. Phase 2 spanned a total of 13 days.

Data Analysis

Independent variable for Phase 1 was the age period in which PCA was conducted: adolescent and adult. Independent variable for Phase 2 were the stressors: environmental and social. Dependent measures for Phase 1 and Phase 2 are the sign- and goal-tracking behaviors during PCA, ethanol intake during PCA, ethanol intake during preference tests, and ethanol preference relative to water during preference tests. The dependent variables in each Phase were analyzed using JASP version 0.11.1. Separate mixed factorial ANOVAs were used followed by additional tests if significant interactions were found (simple main effects, planned comparisons,

or Bonferroni post-hoc comparisons). When Mauchley's tests indicated a violation of the sphericity assumption, degrees of freedom were corrected using the Greenhouse-Geisser estimates of sphericity. Effect sizes are presented as partial eta squared.

Table 1

Experimental Phases and Procedures for Adolescent-trained and Adult-Trained Rats

Weeks in lab	P day at start of week	Adolescent-Trained	Adult-Trained
-	1	Born at Envigo	Born at Envigo
1	21	Enrichment begins	Enrichment begins
2	28	Enrichment only	Enrichment only
3	35	EtOH exposure (2-3%)	Enrichment only
4	42	EtOH exposure (4%)	Enrichment only
5	49	EtOH exposure (5%)	Enrichment only
6	56	PCA Acquisition 1 - 3	Enrichment only
7	63	PCA Acquisition 4 - 6	Enrichment only
8	70	PCA Acquisition 7 - 9	Enrichment only
9	77	PCA Acquisition 10 - 12	Enrichment only
10	84	Enrichment only	Enrichment only
11	91	PCA Acquisition 13 - 15	Enrichment only
12	98	PCA Acquisition 16 - 18	EtOH exposure (2-3%)
13	105	Stressor 1 / Recovery 1	EtOH exposure (4% & 5%)
14	112	Stressor 2 / Recovery 2	PCA Acquisition 1 - 3
15	119		PCA Acquisition 4 - 6
16	126		PCA Acquisition 7 - 9
17	133		PCA Acquisition 10 - 12
18	140		PCA Acquisition 13 - 15
19	147		PCA Acquisition 16 - 18
20	154		Stressor 1 / Recovery 1
21	161		Stressor 2 / Recovery 2

Note. The 'enrichment only' experience in week 10 for the Adolescent-Trained rats was due to technical difficulties necessitating equipment repair.

Results

Phase 1: Acquisition

Sign tracking. Acquisition of PCA sign-tracking behavior for both groups, adolescent-trained and adult-trained, is shown in Figure 2a. Acquisition was conducted for 18 days and plotted as 2-day session blocks, for a total of 9 session blocks. Analyzing and plotting acquisition in blocks of days is a standard in Pavlovian conditioning experiments with many days of training. Sign tracking was measured by the mean total licks per block. A 2 (Group; Adolescent-Trained, Adult-Trained) x 9 (Blocks 1-9) mixed ANOVA revealed a significant main effect of Blocks, $F(8, 112) = 62.90, p < .001, \eta^2_p = .818$, indicating sign tracking increased for both groups over the block sessions, with a large effect size. A Blocks x Group interaction was also found to be significant, $F(8, 112) = 6.00, p < .001, \eta^2_p = .300$. Simple main effects indicated that the groups did not differ significantly at the start of training (Blocks 1 & 2, $p > .30$), but the adult-trained rats displayed statistically significant more sign tracking than the adolescent-trained rats during the middle (Blocks 5 & 6, $p < .01$) and later blocks of training (Blocks 7-9, $p < .05$).

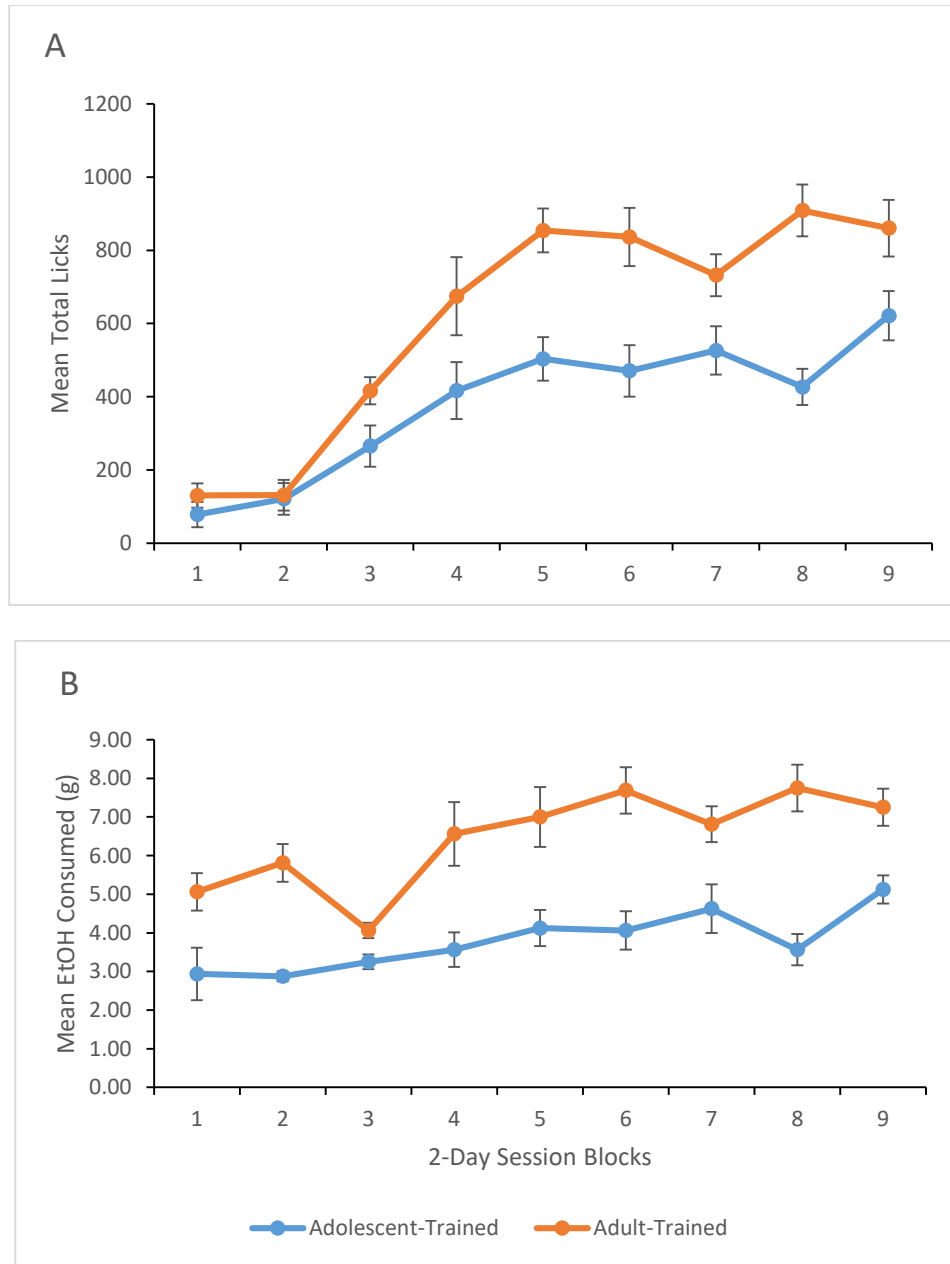


Figure 2. Acquisition of PCA sign-tracking behaviors during the 9 session blocks for both groups, Adolescent- Trained and Adult-Trained. A. Sign tracking is plotted as mean total licks on the bottle per 2-day session blocks. B. Mean consumption of 5% ethanol in grams as the rats were sign tracking the bottle during the 9 session blocks of acquisition. Error bars for both figures are standard error.

Figure 2b displays the mean amount of 5% ethanol in grams consumed during acquisition of PCA sign-tracking behavior. A 2 (Group; Adolescent-Trained, Adult-Trained) x 9 (Blocks 1-9) ANOVA revealed that Mauchly's test of the assumption of sphericity was violated, therefore the degrees of freedom were adjusted using the more conservative Greenhouse-Geisser correction. Throughout the results section the use of this correction is indicated by fractional degrees of freedom with a decimal point. A significant main effect of Blocks, $F(8, 112) = 8.46, p < .001, \eta^2_p = .377$, indicated that ethanol consumption increased for both groups during acquisition. A Blocks x Group interaction failed to reach statistical significance, $F(8.0, 54.8) = 2.49, p = .06, \eta^2_p = .151$. Simple main effects of session block confirmed that both the adult-trained, $p < .001$, and the adolescent-trained rats, $p = .003$, significantly increased their ethanol intake over session blocks.

A comparison of the two age groups' performance during the first days of training (see Figures 2a and 2b, Blocks 1 & 2) shows that while both groups engaged in the same level of sign tracking (Block 1: $p = .30$, Block 2: $p = .87$), the adult-trained rats consumed more ethanol than the adolescent-trained rats (Block 1: $p = .02$, Block 2: $p < .001$). During subsequent blocks of training, the group differences in sign tracking were consistent with the group differences in ethanol consumption. This lick/consumption disparity early in training most likely reflects differences in the form of responding directed towards the sipper bottle. The lick sensor measures a rat's contact with the spout which is usually with the tongue (a lick), but nosing, biting, and paw swipes will also register as contacts. Thus, it appears that early in training the adult-trained rats were interacting with the bottle spout primarily by licking and drinking, whereas the adolescent-trained rats' behavior was more variable resulting in less consumption of

the ethanol. This age difference in response topography early in training will be addressed further in the discussion.

Goal tracking during the bottle CS. Acquisition of PCA goal-tracking behavior for both groups, adolescent-trained and adult-trained, is shown in Figure 3. Goal tracking was measured by the mean total head pokes into the pellet dispenser during the bottle CS presentation for the same 9 total 2-day session blocks. Successful conditioning would be shown by increased head pokes during the bottle presentation (CS) compared to the equivalent amount of time before it is presented (Pre-CS) throughout trials. A 2 (Group; Adolescent-Trained, Adult-Trained) x 2 (Period; Pre-CS, CS) x 9 (Blocks 1-9) mixed ANOVA revealed a significant main effect of Period, $F(1, 14) = 57.83, p < .001, \eta^2_p = .805$ with a very large effect size, indicating that conditioning was achieved for both groups. A significant Group x Period interaction, $F(1, 14) = 6.94, p = .02, \eta^2_p = .331$, was also found. Overall, the adolescent-trained rats head poked significantly more than the adult-trained rats during the CS presentation, $F(1, 14) = 6.61, p = .02, \eta^2_p = .321$, but not during the Pre-CS period, $F(1, 14) = 2.03, p = .18, \eta^2_p = .127$.

A significant three-way interaction, $F(3.08, 43.1) = 4.37, p = .008, \eta^2_p = .238$, was also found; therefore, to evaluate group differences in the acquisition of goal-tracking behaviors, separate Period x Blocks ANOVAs were conducted for each group. These separate ANOVAs revealed significant Period x Blocks interactions for both the adolescent-trained, $F(8, 56) = 4.68, p < .001, \eta^2_p = .401$, and the adult-trained rats, $F(8, 56) = 4.28, p < .001, \eta^2_p = .379$.

Adolescent-trained. Simple main effects revealed that adolescent-trained goal tracking changed over blocks during the CS presentation, $F(8) = 8.00, p < .001$, and the Pre-CS period, $F(8) = 7.66, p < .001$. There were two blocks where adolescent-trained head poking drastically decreased, Blocks 6 and 8. These two blocks included sessions before weekend alcohol

consumption was provided in the home cage overnight, which appears to have reduced head poking in the adolescent-trained rats (but not the adult-trained). However, when these two sessions are removed from analysis, significant simple main effects remain consistent for both CS and Pre-CS periods ($p < .001$). Generally, adolescent-trained head poking during CS presentation increased over blocks. Post-hoc dependent t-tests revealed that adolescent-trained head poking during Blocks 7 and 9 are significantly higher than in Block 1 ($p < .01$). Adolescent-trained head poking during the Pre-CS period was only significantly higher than Block 1 during Block 9 ($p = .03$). Because the rats were tested in their home cages in the colony room there may have been unknown events in the lab (e.g., activity of other researchers or malfunctions with lab equipment) that impacted the adolescent-trained rats' performance during Blocks 6 and 8.

Adult-trained. Goal tracking performance of the adult-trained rats was much more stable than that of the adolescent-trained. Simple main effects confirmed that adult-trained head poking changed over blocks during the CS presentation, $F(8) = 4.64, p < .001$, but not during the Pre-CS period, $F(8) = 1.18, p = .33$. The initial increase in adult-trained head poking during the first 3 blocks demonstrates the acquisition of conditioned goal tracking which was maintained for the remainder of acquisition with a slight decline at the end of acquisition.

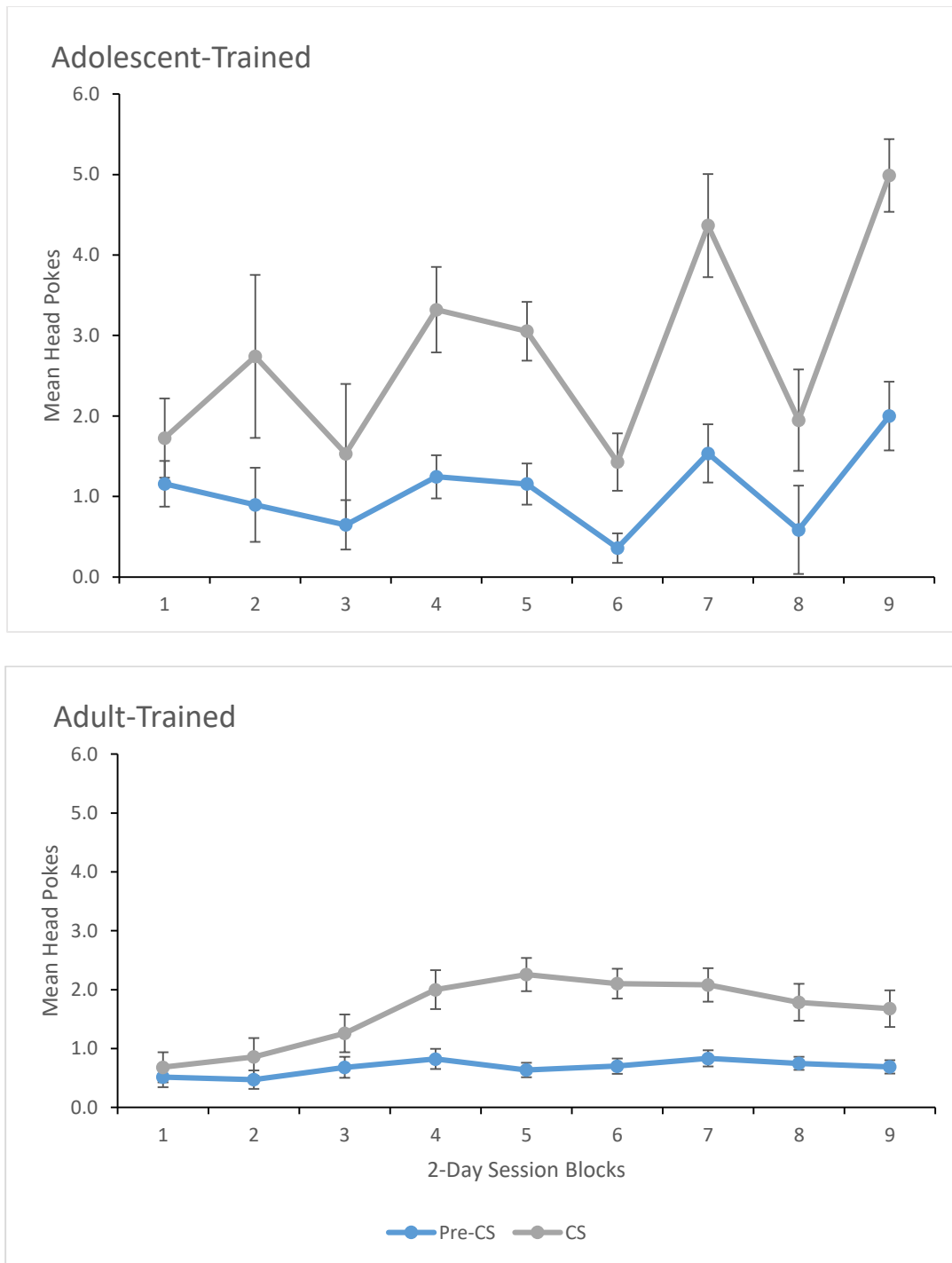


Figure 3. Acquisition of PCA goal-tracking behaviors in the Pre-CS and CS periods during the 9 session blocks for both groups, Adolescent- Trained and Adult-Trained. Goal tracking is plotted as mean total head pokes into the pellet dispenser 10 seconds prior to the CS presentation (Pre-CS) and during the CS presentation (CS). Error bars for both figures are standard error.

Goal tracking during the post-US. Continued head poking into the pellet dispenser following the food pellet presentation (the Post-US period) is typical during conditioning. This measure, which indicates a perseveration of search for food, was compared to the Pre-CS period and is shown in Figure 4 for both groups, Adolescent-Trained and Adult-Trained. A 2 (Group; Adolescent-Trained, Adult-Trained) x 2 (Period; Pre-CS, Post-US) x 9 (Blocks 1-9) mixed ANOVA was conducted. There appears to be a main effect of age with higher Post-US head poking in the adolescent-trained rats than in the adult-trained; however, there was only a trend towards statistical significance with a large effect size, $F(1, 14) = 3.59, p = .079, \eta^2_p = .204$. If Blocks 6 and 8 are removed for this analysis just as in the Pre-CS and CS analysis, the main effect of age becomes significant, $F(1, 14) = 5.29, p = .037, \eta^2_p = .274$. A significant three-way interaction, $F(3.53, 49.46) = 2.96, p = .034, \eta^2_p = .174$, was found, therefore separate Period x Blocks ANOVAs were conducted for each age group. These separate ANOVAs revealed significant Period x Blocks interactions for both the adolescent-trained, $F(8, 56) = 2.32, p = .032, \eta^2_p = .249$, and adult-trained group, $F(8, 56) = 2.71, p = .013, \eta^2_p = .279$.

Adolescent-trained. Including all 9 Blocks, simple main effects confirmed that adolescent-trained goal tracking changed over blocks during the Post-US period, $F(8) = 5.70, p < .001$. However, if Blocks 6 and 8 are again removed from analysis, the simple main effect for the Post-US period is no longer significant, $F(6) = 1.61, p = .169$.

Adult-trained. Simple main effects confirmed that adult-trained goal tracking changed over blocks during the Post-US period, $F(8) = 4.64, p < .001$, similarly to the CS main effect discussed earlier, with head pokes increasing in the earlier blocks to an asymptote and decreasing slightly during the last few blocks.

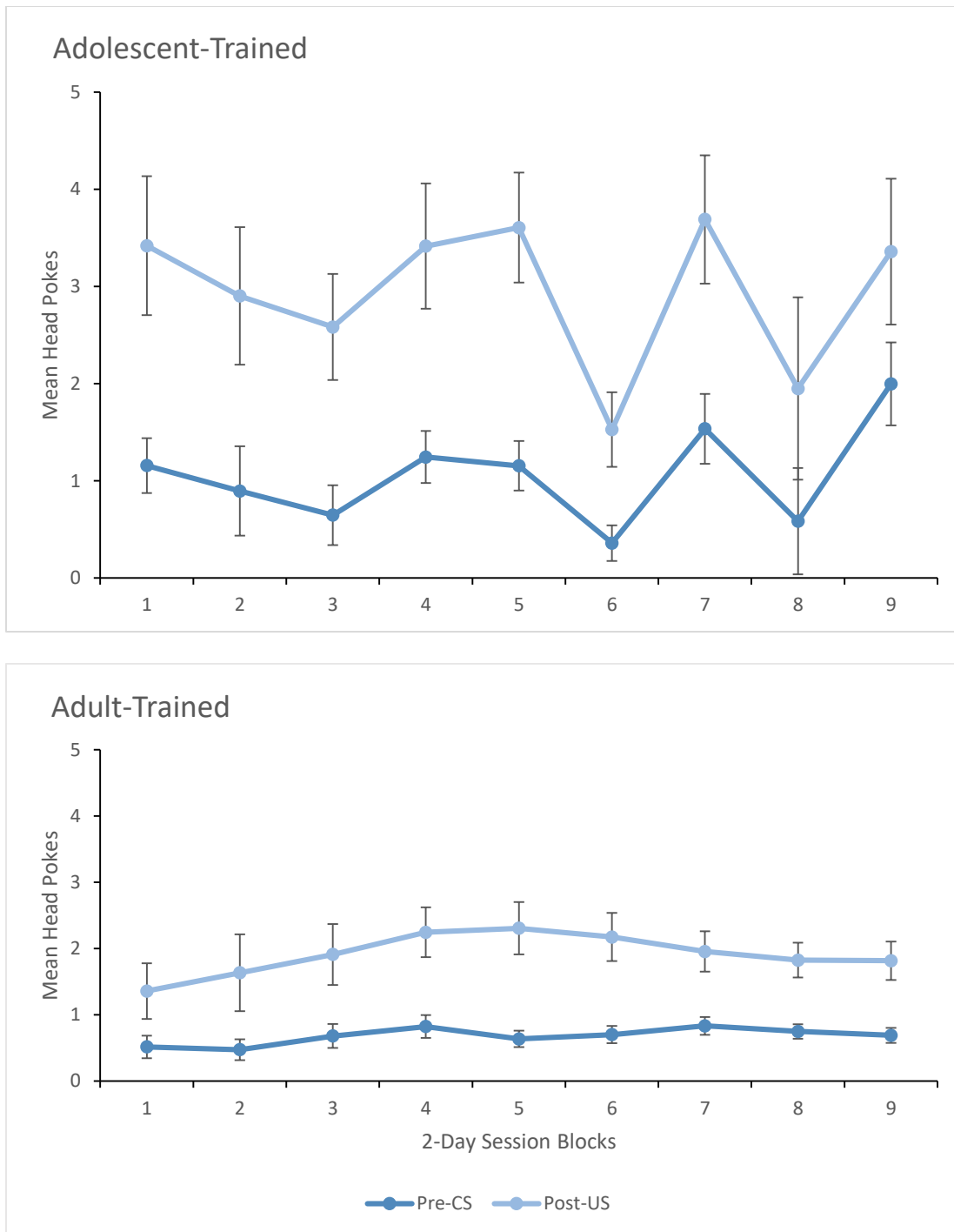


Figure 4. Goal tracking in the Pre-CS and Post-US periods during the 9 session blocks for both groups, Adolescent- Trained and Adult-Trained. Goal tracking is plotted as mean total head pokes into the pellet dispenser 10 seconds prior to the CS presentation (Pre-CS) and 10 seconds following the CS presentation (Post-US). Error bars for both figures are standard error.

Ethanol two-bottle choice tests. Two bottle choice tests were conducted during acquisition of sign tracking in test cages outside of the enriched home environment. The first two tests were during 3% and 4% initial ethanol exposure periods. The remaining four tests were conducted throughout acquisition of PCA.

Ethanol preference. The intakes were computed as percent ethanol consumed and is displayed in Figure 5a. A 2 (Group; Adolescent-Trained, Adult-Trained) x 6 (Sessions 1-6) ANOVA revealed a significant main effect of Session, $F(5.0, 70.0) = 6.65, p = .005, \eta^2_p = .207$; however, when the first two sessions are removed as they were initial exposure periods conducted prior to PCA, this main effect is no longer significant, $F(3.0, 42.0) = 1.49, p = .233, \eta^2_p = .096$. The adolescent-trained rats ($M = 48.96, SE = 23.96$) had an initial lower preference for ethanol during the initial exposure sessions than the adult-trained ($M = 65.88, SE = 9.63$). A trend towards a main effect of Group was found, $F(1, 14) = 4.51, p = .052, \eta^2_p = .243$, however this main effect does not reach traditional levels of statistical significance when the first two initial exposure sessions are removed, $F(1, 14) = 4.14, p = .061, \eta^2_p = .228$. A Group x Session interaction was not found to be significant, $F(5.0, 70.0) = 0.71, p = .621, \eta^2_p = .048$.

Absolute ethanol intake. Figure 5b displays the absolute content of ethanol consumed during the two bottle choice tests. A 2 (Group; Adolescent-Trained, Adult-Trained) x 6 (Sessions 1-6) ANOVA revealed a significant main effect of Session, $F(5.0, 70.0) = 17.89, p < .001, \eta^2_p = .561$ and a significant main effect of Group, $F(1, 14) = 19.31, p < .001, \eta^2_p = .580$. The adolescent-trained rats consumed less ethanol than the adult-trained during the two bottle choice tests for all sessions ($ps < .05$), except the last one $p = .150, d = -0.762$.

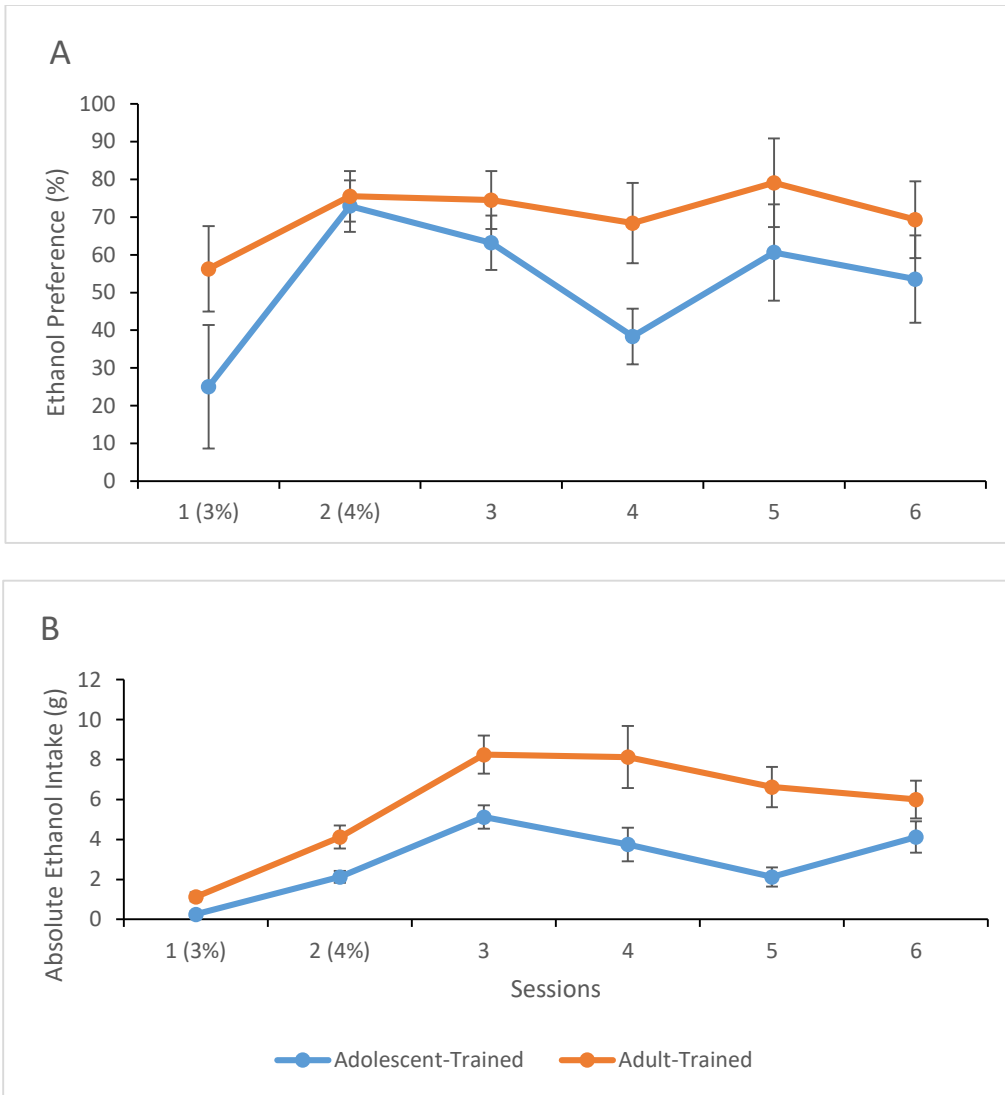


Figure 5. A. Ethanol preference during the pre-exposure sessions, 1 and 2, and sign-tracking sessions, 3 through 6, for each group, Adolescent-Trained and Adult-Trained. Ethanol is plotted as percent intake. B. Absolute ethanol intake during the initial exposure and PCA sessions. Error bars for both figures are standard error.

Phase 2: Acute Isolation Stressors

Licks from the last six sessions of the acquisition trials were averaged to calculate baseline sign tracking performance. Sign tracking under the four stressor conditions (Stressor 1, Recovery from Stressor 1, Stressor 2, & Recovery from Stressor 2) was calculated by averaging the licks from the three sessions of each stressor condition. The same averages were calculated for head pokes (goal tracking).

Sign tracking. The stressor type was counterbalanced within groups to control for order effects; however, several order effects were obtained that complicated the interpretation of the data. Thus, the stressor order was included as a factor in all analyses with all main effects and interactions presented. A 2 (Group; Adolescent-Trained, Adult-Trained) x 5 (Stressor Test; Baseline, Environmental, Return from Environmental, Social, Return from Social) x 2 (Stressor Type Order; Environmental Stressor First, Social Stressor First) ANOVA was calculated, followed by additional ANOVAs when needed.

Main Effects. A significant main effect of Group was found, $F(1, 12) = 8.61, p = .013, \eta^2_p = .418$, with the adolescent-trained rats ($M = 627.95; SE = 57.60$) sign tracking less than the adult-trained ($M = 887.41; SE = 19.70$). A main effect of Stressor Test, $F(4, 48) = 3.65, p = .011, \eta^2_p = .233$, was found with the mean scores as follows: Baseline ($M = 681.30; SE = 152.51$), Environmental Stressor ($M = 748.95; SE = 200.84$), Return from Environmental ($M = 875.85; SE = 30.02$), Social Stressor ($M = 719.72; SE = 142.16$) and Return from Social Stressor ($M = 762.58; SE = 123.12$). Compared to baseline a significant increase in sign tracking was found only during the return from environmental stressor, $p = .019$. A main effect of Order was not found to be significant, $F(1, 12) = 0.12, p = .740, \eta^2_p = .010$.

Interactions. Sign-tracking behaviors displayed at baseline and during the stressor test conditions for both groups, Adolescent-Trained and Adult-Trained, are shown in Figure 6. A Stressor x Group interaction, $F(4, 48) = 2.69, p = .042, \eta^2_p = .183$, was found. Simple main effects revealed that the stressors affected sign tracking of the adolescent-trained rats, $F(4, 28) = 3.79, p = .014, \eta^2_p = .351$, but not the adult-trained, $F(4.0, 28.0) = .45, p = .773, \eta^2_p = .060$. Averaged across order, the adolescent-trained rats ($M = 573.48; SE = 24.17$) sign tracked significantly less than the adult-trained ($M = 882.80; SE = 24.72$) in the baseline measure and during all stressor conditions, $ps < .03$, except the Return from Environmental stressor condition, $p = .73$ (Adolescent-Trained: $M = 845.83, SE = 143.14$; Adult-Trained: $M = 905.88, SE = 92.54$). A significant Stressor x Order interaction was also found, $F(4, 28) = 5.21, p = .001, \eta^2_p = .303$. An increase in sign tracking was seen when the rats were returned to their home after the environmental stressor but only when it was conducted second, $F(4, 28) = 5.10, p = .003, \eta^2_p = .421$; this effect was most likely driven primarily by the adolescent-trained group. A Group x Order interaction was not found to be significant, $F(1, 12) = .244, p = .630, \eta^2_p = .020$.

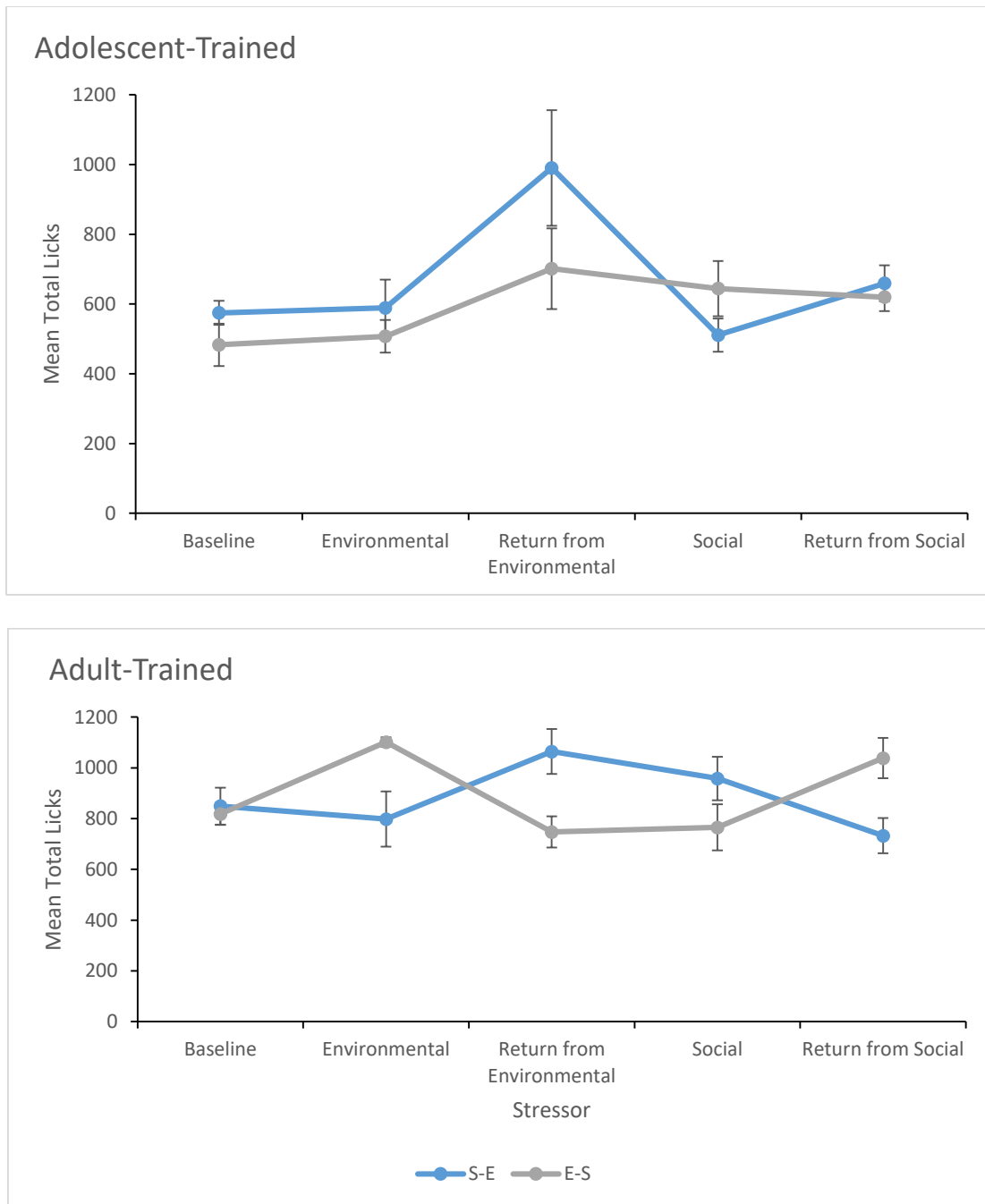


Figure 6. Sign tracking displayed at baseline and during each stressor for both groups, Adolescent-Trained and Adult-Trained. Sign tracking is plotted as mean total licks on the bottle during baseline and each stressor. Error bars for both figures are standard error.

The results reviewed above are qualified by a significant three-way interaction, $F(4, 48) = 3.64, p = .011, \eta^2_p = .233$; therefore, to evaluate group differences in the stressor and order effects, separate Stressor x Order ANOVAs were conducted for each group. These separate ANOVAs revealed a significant Stressor x Order interaction for the adult-trained group, $F(4, 24) = 11.91, p < .001, \eta^2_p = .665$, but not the adolescent-trained group, $F(4, 24) = 1.37, p = .275, \eta^2_p = .186$. The interaction in the adult-trained group is clearly seen in Figure 6. The environmental stressor had the effect of increasing sign tracking during the stressor manipulation when it was the first stressor, but after the return to the home towers when it was the second stressor. A similar pattern occurred with the social stressor condition.

Figure 7 replots sign tracking for the baseline and stressors for each group with stressor conditions shown in the testing sequence that they were presented to better visually capture the order effect. This replotting method will be used for the remaining measures to ease in conceptualizing the overall findings.

Adolescent-Trained. Simple main effects confirmed that the adolescent-trained sign tracking was significantly higher after the return from the first stressor, $F(1, 7) = 5.86, p = .046, \eta^2_p = .456$, regardless of which stressor was conducted first. Despite the large difference in means between the two orders during the return Stressor 2 condition, as noted above, the Stressor x Order interaction was not significant for the adolescent-trained rats.

Adult-Trained. Simple main effects confirmed that sign tracking was significantly higher during the first stressor, $F(1, 7) = 12.94, p = .009, \eta^2_p = .649$, and returning from the second stressor, $F(1, 7) = 15.13, p = .006, \eta^2_p = .684$, regardless of which stressor was conducted second.

A comparison of the two groups' sign tracking performance during the stressors shows a difference in an initial stress effect produced by the first stressor, regardless of stressor type. The

adolescent-trained sign tracking was not affected during the first stressor, meanwhile the adult-trained sign tracking increased significantly. The adolescent-trained sign tracking increased significantly after returning from the first stressor, while the adult-trained sign tracking returned to similar baseline measures. The adult-trained rats were also affected by the return from the second stressor, displaying increased sign tracking, while the adolescent-trained sign tracking remained similar to their baseline measures. These differences will be addressed more in depth in the discussion.

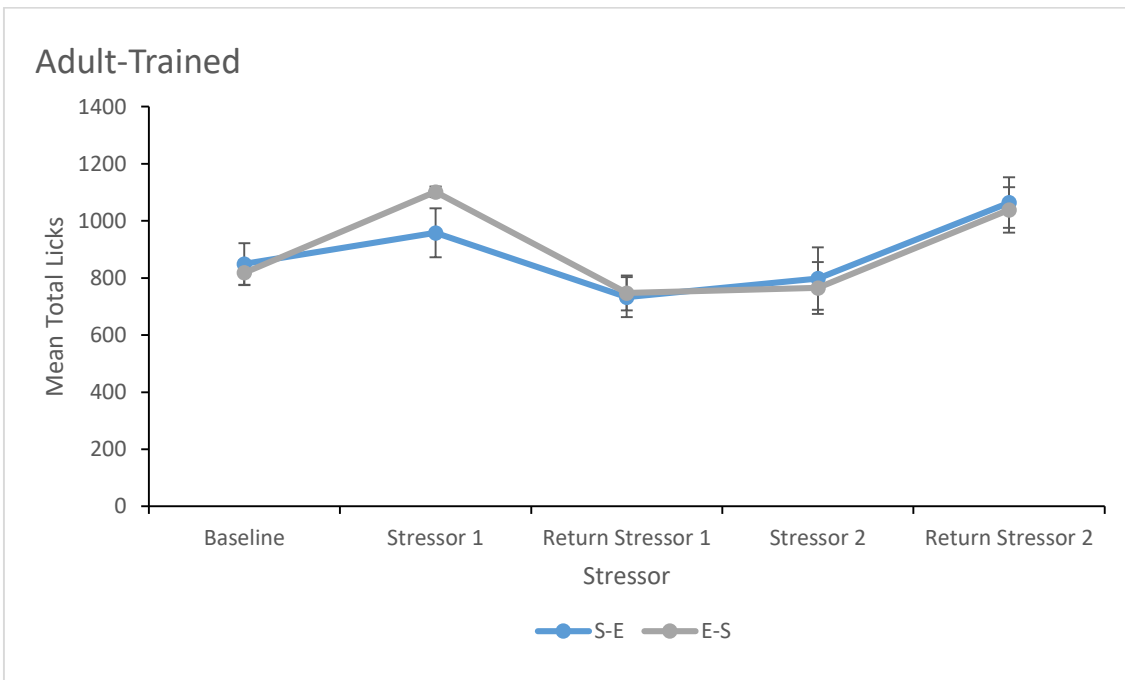
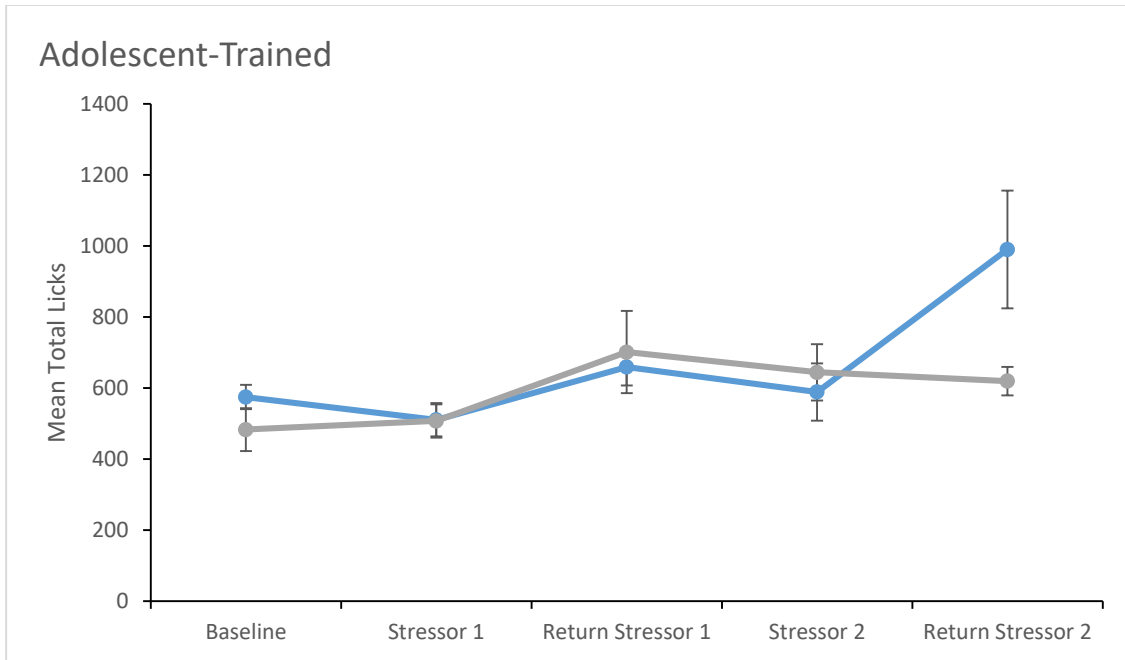


Figure 7. Sign tracking displayed at baseline and during each stressor condition for both groups, Adolescent-Trained and Adult-Trained, organized by testing sequence to display order interaction effects. Sign tracking is plotted as mean total licks on the bottle during baseline and each stressor. Error bars for both figures are standard error.

Goal Tracking during the bottle CS. Total goal-tracking behaviors displayed during baseline and each stressor condition for both groups, Adolescent-Trained and Adult-Trained, are shown in Figure 8 and plotted in the testing sequence that they were presented. A 2 (Group; Adolescent-Trained, Adult-Trained) x 5 (Stressor Test; Baseline, Environmental, Return from Environmental, Social, Return from Social) x 2 (Order; Environmental Stressor First, Social Stressor First) ANOVA was used because of the order effect found in the sign tracking results and to remain consistent throughout the results and discussion sections. A significant main effect of Group did not reach traditional levels of statistical significance, $F(1, 12) = 4.35, p = .059, \eta^2_p = .266$. Nevertheless, the adolescent-trained rats ($M = 224.12, SE = 15.55$) tended to goal track more than the adult-trained ($M = 156.20, SE = 7.16$). Stressor and Order did not achieve main effects, $F(4, 48) = 0.54, p = .705, \eta^2_p = .043$ and $F(1, 12) = 0.33, p = .579, \eta^2_p = .026$, respectively. A Stressor Test x Group interaction was found to be significant, $F(4, 48) = 2.94, p = .030, \eta^2_p = .197$. The adolescent-trained rats goal tracked significantly higher than the adult-trained in the baseline measure, $p = .008$, but this difference is not seen during any of the stressor conditions, $ps > 0.157$. A Stressor Test x Order interaction was not found to be significant, $F(4, 48) = 2.16, p = .088, \eta^2_p = .152$. A Group x Order interaction was also not found to be significant, $F(1, 12) = 0.002, p = .962, \eta^2_p = .000$. A significant three-way interaction, $F(4, 48) = 7.31, p < .001, \eta^2_p = .378$, was found; therefore, to evaluate group differences during the stressors, separate Stressor x Order ANOVAs were conducted for each group.

The separate ANOVAs revealed a significant Stressor x Order interaction for the adolescent-trained, $F(4, 24) = 4.59, p = .007, \eta^2_p = .433$, and adult-trained group, $F(4, 24) = 11.91, p < .001, \eta^2_p = .665$. The interactions can easily be seen in Figure 8 with the replotting method.

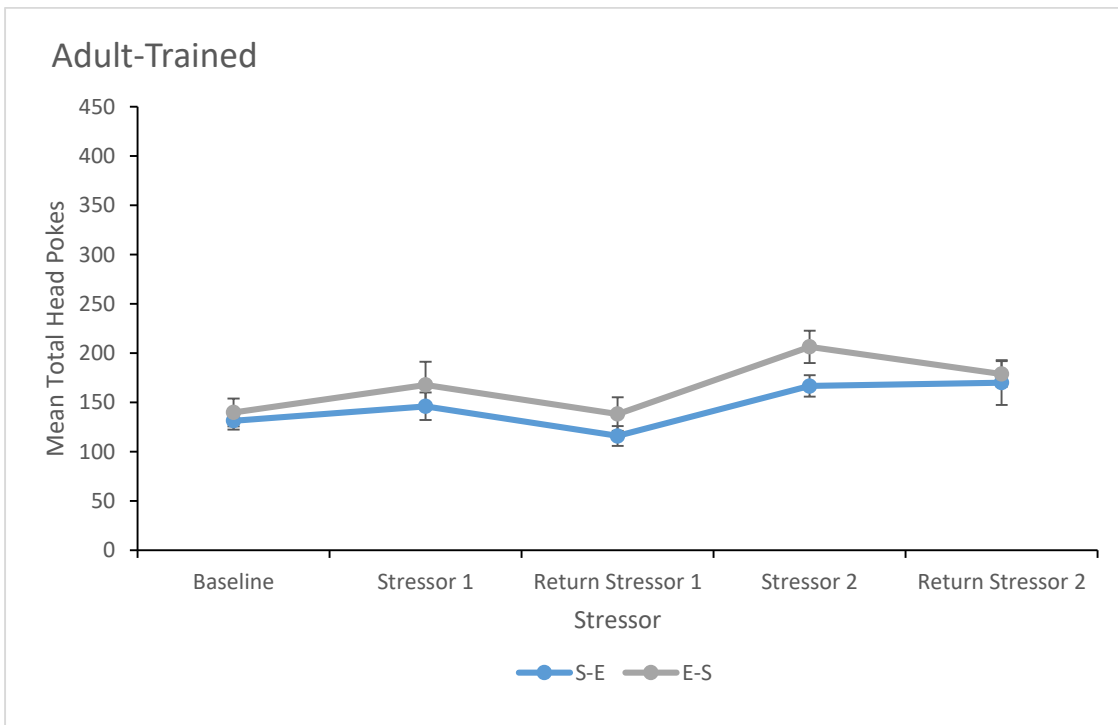


Figure 8. Total goal tracking during sign tracking procedures at baseline and during the stressors for both groups, Adolescent-Trained and Adult-Trained organized by testing sequence to display order interaction effects. Goal tracking is plotted as mean total head pokes into the pellet dispenser throughout all trials of sign tracking procedure. Error bars for both figures are standard error.

Adolescent-Trained. Simple main effects revealed that adolescent-trained goal tracking was significantly lower during the second stressor, $F(1, 7) = 10.32, p = .015, \eta^2_p = .596$, and returning from the second stressor, $F(1, 7) = 10.27, p = .015, \eta^2_p = .595$, regardless of which stressor was conducted second.

Adult-Trained. Simple main effects revealed that adult-trained goal tracking was significantly higher during the second stressor, $F(1, 7) = 34.42, p < .001, \eta^2_p = .831$, and returning from the second stressor, $F(1, 7) = 6.58, p = .037, \eta^2_p = .485$, regardless of which stressor was conducted second.

A comparison of the two groups' goal tracking performance during the stressors shows that as subsequent stressors were issued, a possible chronic stress effect may be seen on goal-directed behaviors which is expressed differently for each group. The adolescent-trained rats goal tracked significantly less after a third dose of stress was administered and continued to goal track less during the subsequent stressor. The adult-trained rats on the other hand goal tracked significantly more following a third dose of stress and continued to goal track at such high levels during the subsequent stressor. This group difference in response to stress will be addressed further in the discussion.

Ethanol consumption. The mean amount of 5% ethanol in grams consumed during PCA sessions during the stressors for each group was analyzed and is displayed in Figure 9. There was an equipment failure during one animal's testing for the return from stressor 2 condition that prevented the ethanol bottle from being fully retracted. To accommodate for this error, the mean of that animal's intakes for the other 2 days of that stressor condition was used. The type of stressor had no effect, therefore the data is presented as a 2 (Group; Adolescent-Trained, Adult-Trained) x 5 (Stress Test; Baseline, During Stressor 1, Return from Stressor 1, During Stressor 2,

Return from Stressor 2) ANOVA. A Group x Stress Test interaction was found, $F(4, 56) = 3.51, p = .013, \eta^2_p = .200$. Simple main effects revealed no significant effect of the stress testing conditions in the adolescent-trained rats, $p = .23$, but a significant effect in the adult-trained, $p = .002$. The two groups differed at all levels ($ps < .05$) except during the second stressor ($p = .21$) due to the adult-trained rats reducing their intake.

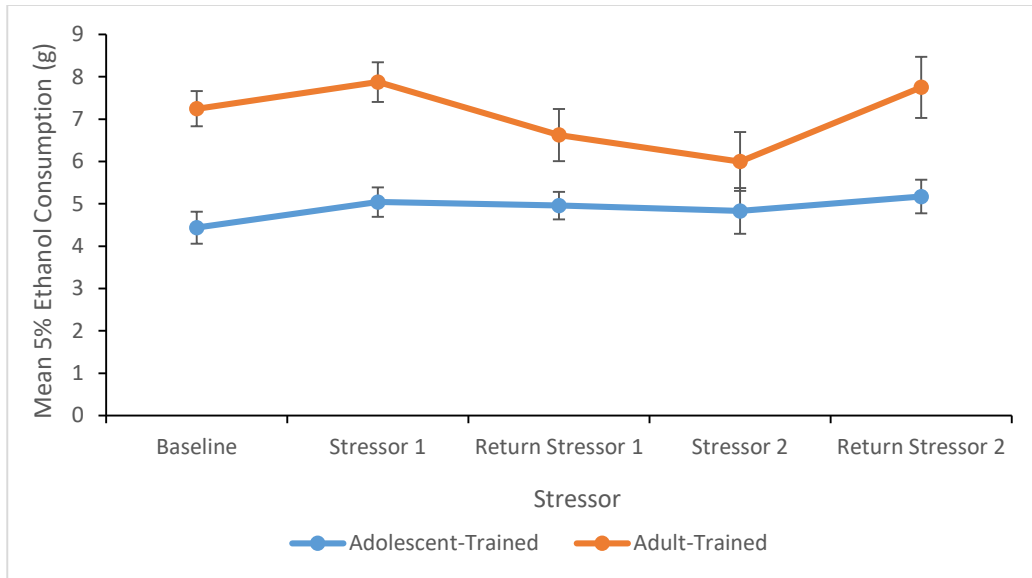


Figure 9. Mean consumption of 5% ethanol (EtOH) in grams in sign-tracking sessions during baseline and each stressor for both groups, Adolescent-Trained and Adult-Trained. Error bars are standard error.

Ethanol two-bottle choice tests. Preference tests were conducted during the stressors after each PCA session. A 2 (Group; Adolescent-Trained, Adult-Trained) x 5 (Stressor Test; Baseline, Environmental, Return from Environmental, Social, Return from Social) x 2 (Order; Environmental Stressor First, Social Stressor First) ANOVA was conducted for ethanol preference and absolute ethanol intake separately.

Ethanol preference. No significant main effects or interactions in ethanol preference during stressors were found. Figure 10a displays the ethanol intakes for each group at baseline and during stressors.

Absolute ethanol intake. Figure 10b displays the absolute intakes for each group. The ANOVA revealed a significant main effect of Stressor, $F(4.0, 48.0) = 13.32, p < .001, \eta^2_p = .526$, main effect of Group, $F(1, 12) = 4.76, p = .050, \eta^2_p = .284$, and a Stressor x Group interaction, $F(4.0, 48.0) = 9.56, p < .001, \eta^2_p = .443$. However, this significance is only seen in the baseline measure, $p = .002$, where the adult-trained rats drank significantly more ethanol in preference tests than the adolescent-trained. This difference is no longer seen in any of the stressors.

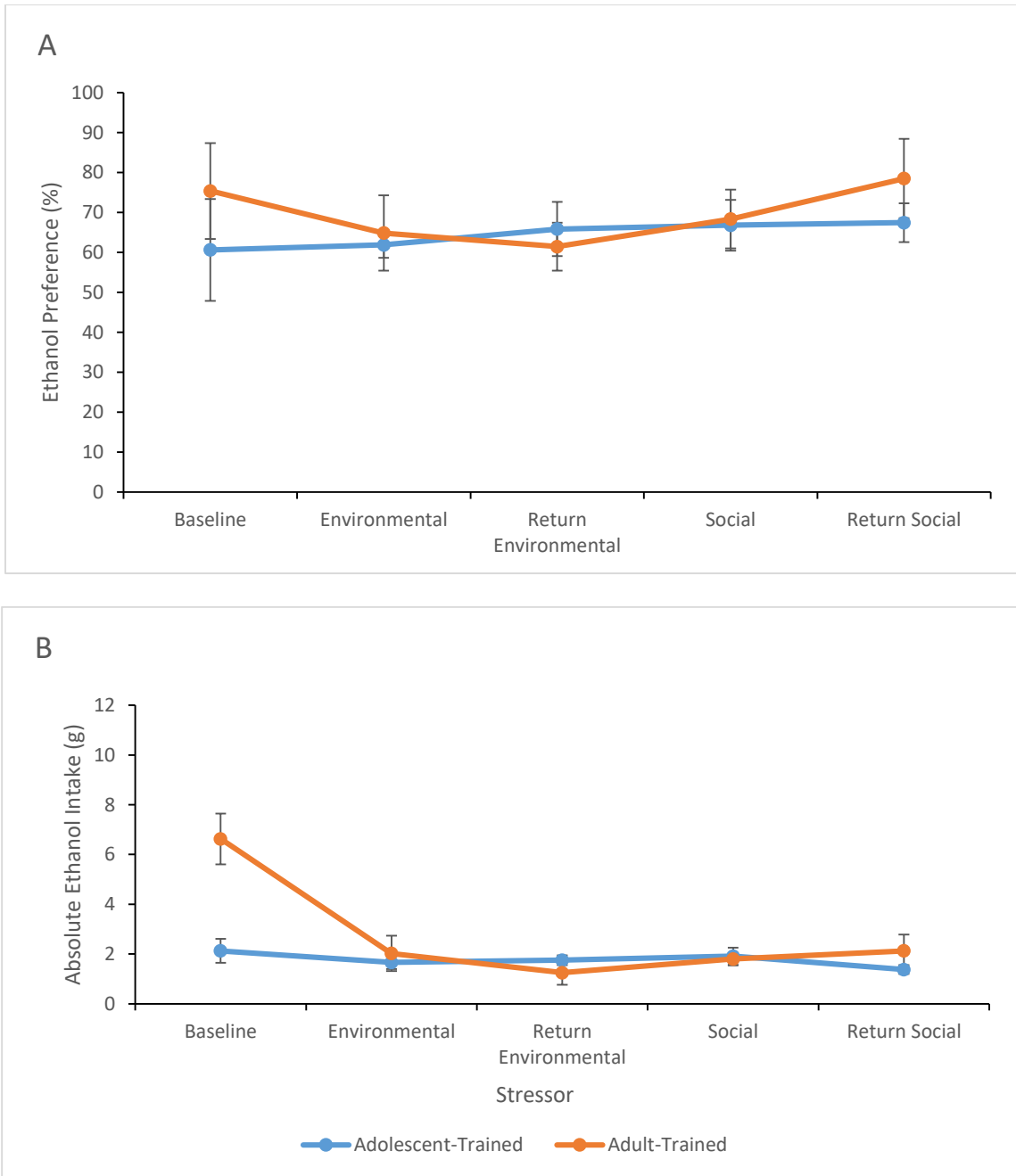


Figure 10. A. Ethanol preference during baseline and each stressor for each group, Adolescent-Trained and Adult-Trained. Ethanol is plotted as percent intake. B. Absolute ethanol intake during baseline and each stressor. Error bars for both figures are standard error.

Discussion

Acquisition of PCA

Sprague-Dawley adolescent rats reared in an enriched environment (EE) displayed less sign-tracking behaviors directed towards a bottle containing 5% ethanol than adult rats who were comparably reared. These significant group differences were not apparent during the beginning of the PCA acquisition phase training. Group differences emerged towards the middle of training and continued to the end of training. This age difference in acquisition of PCA sign-tracking behaviors is consistent with previous findings (Anderson & Spear, 2011; Doremus-Fitzwater & Spear, 2011) even with differences in study design. An important design difference was the use of an ethanol bottle CS in the present study rather than a retractable lever CS. Thus, the age difference seen in the present experiment is unlikely to be due to the ethanol consumed while sign tracking the bottle CS. Ethanol consumption during middle and end of training is consistent with sign-tracking behavior observed during the same bouts of training for both groups, that is the adolescents sign tracked less than adults and therefore consumed less ethanol. However, during the beginning of training, when group differences in sign tracking were not yet observed, adult ethanol consumption was significantly higher than the adolescents, perhaps because the adults were limited in their contact with the spout with licks whereas the adolescent rats interacted with the spout in a more varied way (biting, pawing, licking). Age differences in ethanol consumption have been attributed to the pattern (e.g., involuntary, voluntary, binge, intermittent) and variation of sweeteners (Maldonado-Devincci & Kirstein, 2020). The other substantial difference in procedure was the testing of PCA sign-tracking performance directly in the animals' home environment. This experiment is the first to demonstrate the acquisition of PCA sign-tracking performance in the enriched home environment indicating that sign tracking

is not an artifact of environmentally-restricted rats (i.e., in standard laboratory housing) trained in a separate and distinct test environment (i.e., separate test chambers). Studies using the PCA procedure to induce sign tracking have been done primarily with rats. More recently there has been an interest in translating the insights gained from the PCA procedure in rats to humans (Colaizzi et al., 2020; Schad et al., 2020). By implementing the PCA procedure with an alcohol bottle CS in a home environment that more closely approximates an animal's "natural" state than small restrictive lab cages, the translation of PCA to alcohol consumption in humans has been improved. Although the rats in this experimentally constrained procedure were induced to "voluntarily" drink from an available alcohol bottle, the rats' experience may be similar to human adolescents exposed to "voluntarily" drink alcohol by the constraints of the environment that they find themselves in (e.g., peer pressure to drink and easy access to alcohol).

Most rats engage in both sign- and goal-tracking behavior, therefore I also assessed acquisition of PCA goal-tracking behavior. Goal tracking was operationally defined as the emergence of conditioned head poking. Conditioned head poking behavior is demonstrated when goal-tracking behavior seen during the bottle CS presentation is greater than the Pre-CS period. Both groups rapidly developed conditioned head poking indicating that they were able to predict the US sugar pellet with the bottle CS presentation. However, group differences were apparent throughout all of training with the adolescents' goal tracking significantly higher than adults. This finding is consistent with recent studies reporting greater goal tracking overall (Rode, et al., 2019) and higher locomotor activity (Bishnoi, et al., 2021) in adolescent than adult rats. These results demonstrate that the age group differences in head poking occurred specifically during the CS and not the Pre-CS period, therefore the adolescents were truly goal tracking and increased their search for the food US relative to adults when the food was imminent.

These findings reveal age-specific differences in responding during the acquisition of PCA. One possibility for the adolescent rats sign tracking less than adults is that the adolescent rats may interact with moving objects differently than the adults. With the bottle CS used in the present study, for example, the adult rats may be more likely to treat it primarily as a drinkable source despite its unusual movement in and out of the chamber given their extensive experience with water bottles. The adolescents have less living experience with a bottle than the adults do. Moreover, since adolescence is a time for exploratory (play) behavior (Douglas et al., 2003), the adolescents may be producing more varied behaviors such as pawing or biting the spout rather than consuming its content, particularly because the bottle is a moving stimulus presented in brief trials. This would result in less drinking (i.e., licking) from the bottle prior to frequent training, especially since the rats are not thirsty. This would explain the age difference of ethanol intake in the first 2 training blocks despite no difference in contact with the bottle. The adolescents did eventually increase their sign-tracking behavior and ethanol consumption while sign tracking but not to the levels seen in adults. The adolescents' exploratory behavior can also be seen with their higher levels of goal tracking compared to adults. The adolescents' searching behavior continued throughout the Post-US period. The adolescents' increase in goal tracking is consistent with the claim that adolescents are more responsive to primary incentive stimuli such as food than adults (Rode et al, 2019).

Another possibility for less sign tracking seen in the adolescents could be their sensitivity to alcohol's pharmacological effects (Brown & Tapert, 2004; Crews et al., 2004). It may be possible that the adolescents are initially inclined to reject the consumption of alcohol but eventually throughout training and subsequent exposure are less inclined to do so. Yet again, the adolescents never end up increasing to adult levels with matched experience. Other researchers

reported similar results with a lever CS rather than an ethanol bottle CS used in the present study, suggesting that the ethanol content is unlikely to have been the main cause of the age differences in sign- and goal-tracking behavior seen in the present study.

Some researchers conceptualize sign tracking as habit-like or compulsive-like performance. Perhaps adolescents simply are less prone to habit-like behaviors than adults (Serlin & Torregrossa, 2015) and therefore engage in more goal-directed behavior (Rode et al., 2019). Serlin and Torregrossa (2015) cite neurodevelopmental changes as a reason for the age difference, while Rode and colleagues (2019) suggest differences in susceptibility to develop the habit-like characteristic of sign tracking, which would counteract the learning of behaviors that closely produce rewards, goal tracking. The finding in the present study and elsewhere (Tomie et al., 2004) that rats will reliably consume ethanol compulsively in the presence of reward-paired cues in the PCA environment (sign tracking) but not demonstrate a strong preference for ethanol in a two-bottle test outside of this context is consistent with the concept of a compulsive-like ‘wanting’ of alcohol independent of a ‘liking’ of ethanol. However, recent studies utilizing reinforcer devaluation tests of sign tracking performance suggest that sign tracking is more flexible to environmental contingencies than would be expected of habitual or compulsive responding (Derman et al, 2018; Amaya et al., 2020). Perhaps the sign-tracking behavior that is produced in this paradigm resembles a more flexible compulsive-like action than a strong habit-like response (see Heather, 2017 for varied definitions of compulsive behavior). Conceptualized as a flexible compulsive-like behavior, sign tracking would be expected to be more modifiable through gained experience and changes in sensitivity due to growth in development than habitual responding. Moreover, a compulsive-like behavior may be responsive to stressor effects encountered in the environment in an adaptive or maladaptive manner perhaps depending on age.

For this reason, I tested the sign-tracking behavior of both age groups to acute stressors made possible by my enriched housing setup.

Acute Social Isolation Stressors

After completion of PCA acquisition, isolation stressors were conducted consisting of an isolation from the home enriched environment with a cage mate and isolation from the home environment alone, of which no order effect was found. Only one stressor condition prompted an increase in adolescent-trained sign-tracking behavior, specifically the return from the first stressor (regardless of stressor type). These behaviors were not influenced by change in alcohol preference as two bottle choice tests did not reveal any group or stressor differences.

Collectively, however, the adolescent-trained rats were able to maintain sign tracking levels similar to their baseline. Adult-trained rats' sign-tracking behaviors increased during the first stressor and the last; adult-trained rats sign tracked more when initially encountering a stressor (the first stressor condition) and after four sessions (the return from second stressor condition). Ethanol consumption during training did not change for the adolescent-trained rats either; they were able to maintain their baseline levels throughout the social isolation stressors. The adult-trained rats, however, decreased their ethanol consumption to that of adolescent-trained levels during the second stressor. These findings are consistent with previous literature which found that adolescents were unaffected by acute stress on behavioral measures (McCormick et al., 2008). Adolescent rats were found to exhibit more cognitive and behavioral flexibility than adults (Simon et al., 2013). The present findings suggest that these skills persevered to adulthood and mitigated biological constraints that would sustain increased stress levels.

The present findings are not consistent with a previous study (Anderson et al., 2013) which found that adolescents displayed more sign tracking than adults during stressors. There are

differences in study design which could be mitigating factors, of which the most important are the type of stressor used, when the stressor was implemented, and when acquisition of behaviors was acquired. The present study utilized pair-housing and isolate-housing stressors on rats who were reared in an enriched environment rather than isolate-housing with the addition of food restriction on rats reared in pair-housing conditions. The present study aimed to test the impact of social isolation on previously established sign-tracking and goal-tracking behaviors, not on their acquisition during stressors. The stressors were implemented in adulthood after full acquisition was acquired and when previous literature has found that lasting effects are seen. Anderson et al. (2013) interpreted the adolescents' increase in sign tracking to a possible social buffer acquired through more social interactions based on age. The authors proposed that this social buffer prevented the isolation housing stressor to elicit a significant effect on sign tracking. The present study found significant increases in sign-tracking behaviors for more stressor manipulations in the adult-trained rats than those of the adolescent-trained. The present study's enriched-housing condition would surely produce more social interactions based on the addition of 2 more cage mates compared to pair-housing. Perhaps adolescents are simply less vulnerable to changes in sign tracking generated by stressors after complete acquisition of these behaviors is met in adulthood.

Goal tracking findings revealed no initial changes in behaviors resulting from the first stressor for both age groups. However, the second stressor and return from this second stressor decreased goal tracking in the adolescent-trained rats while increasing the adult-trained levels. Anderson et al. (2013) found that only the food restriction stressor changed goal-tracking behaviors for both age groups, with adolescents increasing and adults decreasing their goal-tracking behaviors; no changes were found from the isolate-housing stressor. The authors

proposed that adolescents are simply more sensitive to food restrictions than adults. Perhaps social isolation stressors only disrupt goal-oriented behaviors if given in succession acutely, such as was found in this study. The adolescent-trained rats' increased goal tracking at the food tray that was seen during PCA acquisition is consistent with the Anderson et al. (2013) results which could be attributed to adolescents being more responsive to food stimuli as discussed in prior section. However, one of the main differences in methodology is that acquisition had already been established prior to the stressor in this study. If adolescents are more responsive to primary stimuli such as food, the change in responding would be attributed to the social isolation stressors and not the acquisition of behavior. Perhaps Anderson et al. (2013) isolate-housing stressor could affect previously established sign- and goal-tracking behaviors; more studies are needed.

The isolation stressor manipulations revealed an interesting possible connection between acute and chronic stressors for adolescent- and adult-learned goal-oriented (goal tracking) behaviors. In the present study, multiple acute stressors were conducted consecutively. The progression of goal tracking results corresponds with previous findings on acute and chronic stress where the initial stressors may be recorded as acute but after multiple occurrences may be experienced as chronic. McCormick and colleagues (2008) found that chronic but not acute social stress increased anxiety-like measures of adolescent rats when they were tested as adults, weeks after the social stressor exposure. This finding indicates a possible residual long-term effect which concurs with the goal-tracking behaviors the adolescent-trained rats exhibited in adulthood. More studies are needed to further investigate the possibility of multiple acute stressors yielding similar results seen from chronic stressors.

The overall findings of the current study do not support the hypothesis that adolescent exposure to the PCA procedure increases vulnerability to compulsive-like alcohol use under acute stress conditions. The adolescent-trained rats were largely able to maintain baseline levels of compulsive-like behaviors, sign tracking, during the stressor manipulations. Nonetheless, the current findings add to the body of literature of several topics. The findings expand on age differences in PCA acquisition and residual effects seen in adulthood. The findings also isolate behaviors elicited from the acquisition of PCA and those from actual stressor manipulations. The possible link between multiple acute stressors and a single chronic stressor was also hinted but requires further investigation. Lastly, the current findings with enriched-housed laboratory rats improve the face validity of the alcohol-bottle PCA procedure as an animal model of human alcohol use and abuse since it results in the emergence of compulsive-like behaviors in a more natural context that also supports a richer behavioral repertoire and concurrent adaptive behaviors. This new animal model allows for studies to investigate the intricacies of age differences on lasting adaptive and compulsive-like learned behaviors and the impact of stressors.

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