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Effect of Environmental Enrichment on the Acquisition of Sign-tracking of an Ethanol Bottle in the Home Environment

Amanda Pra Sisto
amanda.prasisto@student.shu.edu

EFFECT OF ENVIRONMENTAL ENRICHMENT ON THE ACQUISITION OF SIGN-TRACKING OF AN ETHANOL BOTTLE IN THE HOME ENVIRONMENT

By

Amanda Pra Sisto

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College of Arts and Sciences
Department of Psychology

We certify that we read this thesis written by **Amanda Pra Sisto** in the Fall Semester 2020 and, in our opinion, it is sufficient in scientific scope and quality as a thesis for the degree of Master of Science.

_________________________(Date)

Michael Vigorito, Ph.D.

Research Advisor, Seton Hall University

_________________________(Date)

Amy S. Hunter, Ph.D.

Reader, Member of Thesis Committee, Seton Hall University

_________________________(Date)

Kelly Goedert, Ph.D.

Reader, Member of Thesis Committee, Seton Hall University

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Abstract

Sign tracking is a Pavlovian conditioning procedure that has been used in preclinical drug-addiction research with rats to investigate individual differences in the attribution of incentive motivational value to reward cues that may lead to problematic behavior such as drug addiction. Animals demonstrate a tendency to interact with an object conditioned stimulus (CS) that signals the presentation or delivery of a reward, even though the delivery of the reward is independent of any interaction with the stimulus. Thus, it can be argued that sign tracking is a compulsive-like behavior, or an indication of compulsive “wanting” that is associated with increased vulnerability to drug use, abuse, and addiction.

Whereas the dominant approach in the sign tracking literature is to use an arbitrary retractable lever CS and to emphasize the predictive validity of sign tracking of the lever CS on subsequent measures of addictive behavior, in the current study we were interested in employing a CS with greater affordance than an arbitrary lever CS to model compulsive ethanol consumption in Sprague Dawley rats as introduced by Tomie (2014). Moreover, in order to improve the face validity of the traditional sign tracking paradigm as a model of compulsive-like ethanol use and abuse, we raised half of our animals in a more complex or “natural” environment, typically referred to in the literature as environmental enrichment, consisting of small groups of rats in multilevel towers with access to objects and toys. Unlike previous studies, we also tested the animals in these enriched towers rather than in separate testing chambers.

We found that rats in the environmental enrichment condition acquired sign-tracking behavior at a significantly faster rate and approached a higher asymptote than standard-caged rats. Testing within a home environment did not diminish sign-tracking activity, suggesting that sign tracking is not dependent on impoverished rearing and isolated test chambers and can

emerge in a familiar setting. By testing rats within their home towers and extending the period of sign tracking beyond that of past studies, we have contributed to a limited body of pre-existing research on the influence of environmental enrichment on sign tracking and further validated Tomie's model of sign-tracking of an ethanol bottle as an animal model of excessive and compulsive-like use and abuse.

Introduction

Alcohol has been consumed by humans for centuries and continues to be a popular drug worldwide, as it is known to increase relaxation, elevate mood, and facilitate socialization (Meyer & Quenzer, 2018). The fact that alcohol is legal and readily available in America may contribute to why it is one of the most commonly used psychoactive drugs in the country, as well as the most abused. According to the 2017 National Survey on Drug Use and Health (NSDUH), 19.7 million Americans age 12 and up suffered from a substance use disorder that year (SAMHSA, 2018). Out of these individuals, 74% were battling an alcohol use disorder. Repeated exposure to alcohol can result in tolerance, whereby physiological changes occur in order to adapt to or counter the effects of the drug (Julien et al, 2010). In any case of drug tolerance, the user must take greater amounts of the drug in order to achieve the same effects. Chronic alcohol use leads to physical dependence, at which point the user experiences withdrawal symptoms after drinking stops.

Alcohol exerts its psychoactive effects by acting on multiple neurotransmitter systems including glutamate, GABA, dopamine, and opioid systems (Tomkins & Sellers, 2001). Alcohol causes the release of dopamine in the nucleus accumbens upon activation of dopaminergic neurons in the ventral tegmental area of the brain. This mechanism is believed to be responsible for the reinforcing effects of alcohol (Olivia & Wanat, 2016). In response to heavy or prolonged alcohol use, the brain will produce less dopamine or reduce the amount of dopamine receptors available (NIDA, 2018). Therefore, a person is less able to experience pleasure from not only the drug but natural rewards as well. Some theoretical accounts of drug and alcohol abuse emphasize the role of dopamine in the maintenance of behavior through positive and negative reinforcement (e.g., operant self-administration studies). Another theoretical approach also acknowledges the involvement of dopamine but emphasizes its role in the development of *incentive salience* through associative learning (i.e., Pavlovian conditioning).

Incentive salience is a type of "wanting" that is typically triggered by reward cues, which may include visual, auditory, or olfactory stimuli associated with the reward (Berridge, 2012). This type of "wanting" has been referred to as a "compulsive urge" driven by learned cues and is different from cognitive wanting, which is desire driven by an explicit goal (Berridge, 2012, Berridge & Robinson, 2016). Incentive salience "wanting" is also independent of "liking," although the two have historically been tied together (Berridge & Robinson, 2016). Whereas "wanting" is an induced motivation to seek out and interact with a substance such as alcohol, "liking" is the preference of an alcoholic drink over a non-alcoholic drink when given a choice. "Liking" refers to the actual pleasure derived from the reward.

Incentive salience "wanting" is mediated by the mesocorticolimbic system and the release of dopamine to brain regions such as the nucleus accumbens (Berridge & Robinson, 2016). In contrast to this large and robust network, the system that mediates "liking" is more fragile and is concentrated within small hedonic hotspots. Dopamine has been found to enhance salience wanting, but not liking, of a substance. This theoretical distinction between "wanting" and liking has led to the *incentive sensitization theory of addiction*, which states that addiction reflects amplified "wanting" when reward cues are present, without necessarily being accompanied by increased liking (Berridge & Robinson, 2016). Therefore, someone living with addiction may experience incentive salience wanting triggered by reward-related cues, even if he or she has no cognitive desire for the substance or does not expect the substance to be pleasurable. There is evidence that amplified "wanting" is the result of neural sensitization, or "long lasting changes in dopamine related motivation systems" (Berridge & Robinson, 2016, p.

1). Interestingly, some individuals seem to be more susceptible to incentive sensitization than others, making them more likely to engage in excessive drug and alcohol intake.

Animal Models of Alcohol Use and Abuse

The severity of the widespread and persistent problem of alcohol abuse and addiction warrants research into unanswered questions and possible preventative measures. Understandably, it could be a breach of ethics to administer alcohol to humans chronically in lab settings and therefore, the use of animal models has been imperative in unveiling some of the neural mechanisms and behaviors that characterize abuse and addiction. Access to nonhuman animal species has allowed researchers to comprehensively review patterns that lead to drug preference and excessive intake of a substance.

A challenge of using animal models of alcohol abuse is that rodents do not typically self-administer alcohol when given an opportunity to do so (Becker & Ron, 2014). In experiments studying the pharmacological or behavioral effects of alcohol in lab animals, consumption can be forced by pumping ethanol directly into the stomach. However, voluntary consumption is a critical component for methods designed to replicate substance abuse. One way of inducing voluntary intake is by mixing ethanol with sucrose to create a sweet tasting substance. Other recent and effective ways involve schedules. For instance, it has been found that when alcohol is reintroduced following periods of deprivation, animals are more likely to self-administer and even excessively consume (Becker & Ron, 2014). Another schedule-dependent paradigm, known as sign tracking, relies on repeated pairings of ethanol and a food reward in order to instigate drinking.

Pavlovian Sign tracking Procedure

Sign tracking is a Pavlovian conditioning procedure that can be used to induce substance intake in rodents without relying on forced consumption. Animals demonstrate a tendency to interact with stimuli that signal the availability of a reward (Domjan, 2006). The signaling stimulus itself is not reinforcing or rewarding, and the delivery of the reward is independent of any interaction with the signal.

The earliest sign-tracking experiment was conducted by Brown and Jenkins (1968) who exposed pigeons to the presentation of a key light immediately followed by the delivery of a food reward. The repeated pairing of these two stimuli resulted in an interesting behavior by the pigeons; they reliably responded to the key light by pecking at it prior to food delivery even though responding was unproductive and unnecessary for reward delivery. The authors speculated that the emergence of this behavior might have been an indication of classical conditioning. This conditioning effect can be so strong that animals will give up the opportunity to obtain the reward in order to respond to the signaling stimulus (Hearst & Jenkins, 1974).

The behavior the pigeons displayed in the earliest experiments has come to be known as "sign tracking," since they appeared to be tracking the light as a signal for the reward to come. These types of experiments have continued to be carried out and have expanded to include rodent subjects. The sign-tracking behavior observed in these studies has also been described by some researchers as "conditioned approach behavior" (Fitzpatrick & Morrow, 2016). Most often for rat studies, a lever or a water bottle acts as a motivationally "neutral" conditioned stimulus (CS). A sugar or food pellet is employed as the reward, or the unconditioned stimulus (US). The US is delivered regardless of whether interaction with the CS occurs; however, some rats direct their responses, which may include licking, chewing, or sniffing, at the CS (Tomie & Sharma, 2013). One interpretation of this behavior is that as a signal for the reward, the initially neutral CS gains its own incentive value, driving the sign-tracking behavior (Morrison et al, 2015). This can be compared to how addicts attribute incentive salience to drug-related cues, according to the incentive sensitization theory of addiction described previously. Drug seeking and drug taking by addicts has been described as a “compulsive” behavior that may be driven by the presence of environmental stimuli closely related to the drug reward (Everitt & Robbins, 2006). It can be argued that sign tracking is also a compulsive behavior. For instance, rats that are presented with a water bottle CS paired with a sugar pellet US will eventually begin to lick the bottle and drink the water, even though they are not thirsty. This action does not appear to provide any direct benefit to the animals, since the reward delivery is not contingent upon interaction with the CS, and yet, the rats cannot seem to resist the urge to drink. A consensus appears to be growing in the field of Behavioral Neuroscience that this behavior is driven by the attribution of incentive salience to the water bottle CS (Berridge & Robinson, 2016).

If the sign-tracking paradigm is, in fact, a valid model of compulsive-like behavior, it is possible to induce rats to compulsively consume not just water, but other substances such as ethanol. Ethanol, or ethyl alcohol, is the same type of alcohol found in beverages commonly consumed by humans. Ethanol functions as an effective CS, since repeated pairings of ethanol with a rewarding US have been shown to induce sipper-directed responses and ethanol consumption (Tomie & Sharma, 2013).

There is evidence that alcohol can act as both an effective CS and US. When a lever was presented as the CS and alcohol as the US, rats tended to lever press in anticipation of the alcohol presentation (Tomie & Sharma, 2003). In another experiment, rats receiving an ethanol bottle as a CS during a sign-tracking procedure showed greater fluid consumption than those receiving a water bottle as the CS (Tomie et al, 2004). This could suggest that the poorly controlled habit of interacting with a predictive CS combined with the pharmacologically rewarding effects of alcohol leads to excessive ethanol intake in rats undergoing these procedures.

Like animals, humans also tend to attribute incentive salience to reward cues. Sensory stimuli, such as smell and taste, can act as predictors that signal alcohol (Srey et al, 2015). Such stimuli can induce a conditioned response, which is excessive intake. A variety of items can act as CSs to evoke drinking, including glassware, alcohol brand, and perhaps even context. A person's favorite bar, for example, may serve as a discriminative stimulus or "occasion setter" because it is associated with alcohol rewarded stimuli and behavior. Therefore, the person may develop a tendency to drink excessively in that location, as opposed to a novel context. Alcohol may also serve as a CS for humans, as in animal sign-tracking procedures. Alcohol is usually consumed while enjoying entertainment or relaxation, and often in the company of others. Therefore, alcohol itself can act as a reward cue signaling these favorable situations.

It is important to note that during sign-tracking training, not all animals display sign-tracking behavior. *Goal tracking* is another phenotype, which is characterized by the animal approaching the location of the reward rather than the CS while the CS is present. For goal trackers, the CS does not gain incentive salience as it does for sign trackers, and therefore does not become a desired or attractive stimulus (Robinson & Flagel, 2009; Robinson et al, 2014). After repeated training, goal-tracking begins to diminish in some animals while sign-tracking behavior takes over, indicating that the CS gradually gains incentive value (Srey et al, 2015). Other times, goal tracking or a mix of goal-tracking and sign-tracking may be observed for the entire duration of training. It has been suggested that because sign-trackers attribute greater incentive salience to reward-related cues than goal trackers, they are more vulnerable to substance abuse and addiction-related behavior (Fitzpatrick & Morrow, 2016). The fact that individual differences exist within the sign tracking paradigm is good for an animal model of alcohol use, since alcohol consumption varies greatly in humans too. Obviously, not all humans who consume alcohol become addicted or abuse the substance (Esser et al, 2014). Furthermore, it is possible to explore these individual variations through experimental manipulations, which may differentially affect the acquisition of sign tracking.

Intermittent presentations

Another way to induce ethanol intake in lab animals without forcing feeding or mixing with sweet solutions is through intermittent access. Typically, a two-bottle choice procedure is implemented, so that the rat is presented with both water and ethanol for set time intervals (Carnicella et al, 2014). Therefore, the alcohol is not always available to the animal. With this method of repeated periods of 2-bottle choice and withdrawal, it may take several weeks of training before high amounts of ethanol are consumed. Interestingly, only a small percentage of subjects reach a pharmacologically relevant blood ethanol content (BEC) during these procedures, indicating that again, there may be underlying individual differences in excessive voluntary ethanol seeking behaviors and consumption (Carnicella, et al, 2014).

The sign tracking procedure induces ethanol intake without explicit schedules of operant reinforcement, supporting the view that ethanol-drinking behavior is a result of learned incentive motivation. The intermittent 2-bottle access procedure has been primarily interpreted as resulting from reinforcement processes (i.e., negative reinforcement set up by periods of ethanol withdrawal). The intermittent access procedure also results in an increased preference or “liking” for ethanol (Carnicella et al, 2014). While the sign tracking of alcohol is believed to increase the incentive value ("wanting") of the ethanol bottle, the procedure does not consistently result in a preference (liking) for ethanol over water (Tomie et al, 2004, Casachahua, 2011).

Environmental factors in individual vulnerability

Out of all individuals that self-administer a given drug with abuse potential, only a small percentage will become addicted (Esser et al, 2014; Meyer & Quenzer, 2018). There are many factors that appear to play a role in individual susceptibility to substance abuse and addiction. One is genetic variation, for example, as indicated by the success in selectively breeding for alcohol-preferring rats (Ciccocioppo, 2013). Others include environmental influences, such as stressful life events, education level and employment, and being surrounded by substance-using peers (Meyer & Quenzer, 2018). Different environmental situations can be replicated as animal models in order to better understand their influences on drug-taking and seeking behavior.

In an experiment conducted by Kulkosky et al. (1980), rats that were housed in a natural habitat consisting of three males and three females in a space filled with dirt, rocks, and trees consumed significantly less total ethanol than rats housed in isolation or in a group cage. The authors speculated that this effect may have been due to the reduced stress of the natural-housed rats in comparison to the crowding or isolation groups. Another early experiment conducted by Alexander et al (1981) produced similar findings, although morphine was used rather than ethanol. Rats housed socially and in an enriched environment self-administered significantly less of a morphine solution than did isolated rats. The results of a study conducted several years later suggest that in addition to current housing conditions, the environment in which an animal was reared may also have an influence on alcohol intake (Rockman et al, 1989). In this experiment, rats that were reared in an enriched environment for 90 days and continued this type of housing into adulthood consumed significantly greater amounts of ethanol than rats reared and housed in individual cages. Moreover, this same group consumed more ethanol than rats that were reared in an enriched environment for 90 days and then placed in isolation as well as rats that were reared in isolation then transferred to environmental enrichment. Note that in this study, consistent exposure to enriched housing increased rather than decreased alcohol use.

Studies that have reported reduced drug self-administration in rats raised in an enriched environment have contributed to the prevalent idea that environmental enrichment is a protective force against drug and alcohol abuse (Stairs & Bardo, 2009). A recent review concluded that enrichment provides a degree of cognitive stimulation and modulatory control on anxiety and impulsivity that may reduce the transition to compulsive abuse and addiction (Rodriguez-Ortega & Cubero, 2018). However, as is apparent in the Rockman et al (1989) study mentioned above, there have been contradictory findings that suggest the opposite. Some researchers have found that environmental enrichment rats are more sensitive to the rewarding effects of drugs such as amphetamine (Green et al, 2010, Bardo et al, 1999). This should, in theory, make these rats more vulnerable to excessive consumption of the drug.

In light of the large number of studies focusing on the impact of environmental enrichment on drug consumption, very little have employed the intermittent access procedure as a method of self-administration in rats. Even less have used the sign-tracking paradigm. There is only a limited number of studies that have examined the effects of environmental enrichment on sign-tracking behavior. Beckmann and Bardo (2012) found that rats reared in an enriched environment with large cages, social cohorts, and novel objects tended to maintain goal-tracking behavior while rats reared in isolation primarily displayed sign-tracking behavior during a sign-tracking procedure with a retractable lever CS. Unsurprisingly, the authors speculated that the enriched rats attributed less incentive salience to the reward cue (the lever) than did the rats reared in isolation. Therefore, environmental enrichment may reduce the tendency of an individual to attribute this motivational value to reward-related cues (Beckmann & Bardo, 2012).

These conclusions contradict those of another study in which environmentally enriched rats trained to sign track a water bottle CS acquired sign tracking at a *stronger* rate than did pair-housed rats in standard cages (Casachahua, 2011). In addition to the differences between the CS (lever vs. water bottle), the two studies differed in other procedural details. For example, sign-tracking sessions were carried out for twice as many days (10 days) in the Casachahua study than in the Beckmann and Bardo study (5 days). As mentioned previously, goal-trackers have a tendency to switch to sign tracking over repeated training sessions (Tomie & Sharma, 2013), so a longer duration of sessions is likely to reveal more sign tracking. Besides these two experiments with conflicting findings, there appear to be no other published studies that have looked at the impact of environmental enrichment on sign tracking. While environmental enrichment and schedule-induced effects are typically investigated separately, the two manipulations will be combined in the current study.

Summary and Purpose of Current Experiment

The question of the current study was whether environmental enrichment differentially affects sign tracking acquisition as opposed to standard rearing. If the tendency to assign incentive value to signals for reward increases vulnerability to excessive drug use and abuse (Tomie, Grimes, & Pohorecky, 2008; Robinson et al, 2014) it is important to know if animals reared in an enriched environment are less or more vulnerable to compulsive drinking induced by the sign-tracking procedure. The current study differs significantly from the Beckmann and Bardo experiment (2012) described previously in that we selected to use an ethanol sipper as our conditioned stimulus, which offers greater affordance than the lever. Furthermore, we allowed sign-tracking behavior to develop over the course of 18 sessions, extending the duration of this phase well past the five sessions allotted in the Beckmann and Bardo study.

Furthermore, a novel aspect of our experiment is that sign-tracking sessions were run in the home towers that housed the enrichment group. To our knowledge, this has never been done in the past, as studies investigating schedule-induced behavior change in animals are routinely conducted in test chambers separate from the home cage.

It is well established that context plays a modulatory role on conditioned behavior (Holland, 1992). There is evidence that behavioral sensitization effects induced by stimulant drugs (e.g., amphetamine-induced locomotor sensitization) are modulated through associative learning by context that serve as "occasion setters" (Anagnostaras, Schallert, & Robinson, 2002). Similar neural sensitization processes are assumed to be involved in the incentive sensitization process which is believed to control sign tracking. If sign tracking emerges only under strong contextual control, then the phenomenon should be constrained by the testing procedure. To improve the face validity of an animal model of excessive drug use and abuse we upheld that animals should be tested in their own home environment, rather than in an isolated test chamber. This would inform us whether a separate distinct context is necessary to develop and maintain sign-tracking behavior or whether sign tracking also emerges in a familiar context associated with more than just the sign tracking procedure.

In the current study, 21-day old rats were randomly assigned to either standard paired housing or environmental enrichment housing with four cage mates. After about five weeks of exposure to their environments, adolescent rats underwent sign tracking sessions in the environmental enrichment towers. For the enriched housed rats, the training occurred in their familiar home environment, whereas for the standard-housed rats the training was conducted in a distinctly separate training environment. Sugar pellets were selected to serve as the US while a sipper containing 5% ethanol solution acted as the predictive CS. Sign tracking was continued for six weeks for a total of 18 sessions per subject. Sign-tracking and goal-tracking behavior were measured by interactions with the CS and US locations, respectively. The volume of ethanol consumed during sign tracking was recorded, as well as ethanol consumed during periodic two-bottle preference tests.

The primary question was whether there would be a difference between environmental enrichment rats and standard pair-housed rats in the acquisition of sign tracking. The effect of testing within a home context had yet to be explored until now, so it was unclear what effect this would have on the dependent variables. In standard sign tracking procedures in which rats are removed from the home cage and tested in a separate sign-tracking chamber, context acts as a discriminating stimulus or “occasion setter” that may modulate sign-tracking behavior. Therefore, when the standard caged rats are placed in the tower for sign-tracking (while the resident rats are removed from the tower), contextual cues, such as the mesh floor and walls act as discriminative stimuli that signal that it is time for sign tracking. If the emergence of sign tracking requires a salient and distinct discriminative stimulus, training in the home cage for the enriched group should reduce the contextual control of sign tracking, since the home context is not exclusively paired with sign tracking. In fact, the home environment is associated with the execution of *all* daily activities, like sleeping, eating, and playing. Therefore, the emergence of conditioned drug seeking behavior in animals tested within their home cage would suggest that this compulsive-like behavior also emerges in a complex environment and is not strongly modulated by an occasion setter. This finding would be consistent with the results of a recent study that found that rats sign tracking a retractable lever were less sensitive than goal trackers to the modulating effects of an occasion setter (Ahrens et al, 2016). Demonstrating the emergence of sign tracking of an alcohol-containing bottle in a complex home environment will improve the face validity of sign tracking as an animal model of compulsive behavior and compulsive alcohol use for translational research.

The face validity of the sign-tracking model was further improved in the current experiment by including an enriched experience for the rats in the towers. It is well known that operant drug self-administration is reduced in rats (Craig et al, 2016) and people (e.g., Hart et al, 2000) when alternative non-drug reinforcers are made available in the training context to compete with the drug reward. This finding suggests that the demonstration of drug use in laboratory animals is dependent on the lack of alternative reinforcers in the test chamber. Studies also demonstrate that greater opportunity for alternative behaviors provided by enriched housing reduces operant self-administration that takes place in separate test chambers (Yates et al, 2019). If classically conditioned sign-tracking behavior also depends, at least partly, on a test and home environment that does not provide opportunities for alternative behaviors then it may be expected that sign tracking would be reduced in the enriched rats compared to standard housed rats. Alternatively, given that enriched housing has been found to increase exploratory behavior (Modlinska et al, 2019) and to enhance learning (and associated changes at the level of the synapse) (Hullinger et al, 2015), we posited that greater investigatory contact with the tangible CS might increase sign tracking behavior in the enriched rats relative to standard housing controls.

Returning once more to the incentive-sensitization theory of addiction, sign-tracking behavior in this experiment indicated “wanting” of the ethanol. This is different from liking, which refers to the reinforcing effects of the taste and the pharmacological effects of the alcohol.

“Liking” was measured by occasional two-bottle choice tests conducted outside of the home context. We expected that rats displaying strong sign-tracking behavior would not necessarily show a preference (liking) for ethanol, based on results from past studies that have supported the notion that these two states are not linked (Tomie et al, 2004, Casachahua, 2011). In conclusion, our intention for this study was to see whether housing condition (environmental enrichment vs. standard housing) affects the relationship between “wanting” (sign tracking behavior) and liking (preference tests) of alcohol in an experimental setup that at face value more closely approximates the human condition than traditional laboratory procedures.

Method

Subjects

Sixteen male Sprague-Dawley rats purchased from Envigo at 20 to 21 days of age were used in this study. The animals were given ad-libitum access to food (Teklan rodent diet, 7102) and tap water throughout the experiment, except where noted in the procedure. A 12:12 hour light-dark cycle was maintained, and all testing occurred during the light cycle. We obtained IACUC approval for this experiment prior to data collection.

Design

Each rat was randomized into one of two groups immediately upon arrival to the laboratory. Half of the rats (n=8) were randomly assigned to the enrichment group and placed into one of two towers, for a total of four rats per tower. The rest of the rats were randomly split into pairs and divided into four smaller cages for standard housing. Rats remained in their respective conditions for the duration of the study.

We used a repeated measures design, where group (standard versus environmental enrichment) was the between subjects factor, and training sessions (18 sessions split into nine 2-day blocks) was the repeated measures factor. Our dependent variables were licks to the ethanol sipper, total volume of ethanol solution consumed, and average number of headpokes into the sugar pellet delivery site during sign tracking sessions. For headpokes, we also extracted the number of headpokes during the CS period and the number of headpokes during the pre-CS period for an additional repeated measures factor.

In addition to sign tracking, we conducted a total of four individual two-bottle choice tests, two in the beginning weeks of sign tracking and two following week five. Our dependent variables were absolute volume of ethanol consumed, as well as preference for ethanol versus water.

Materials

Two towers made of 1/2" x 1" galvanized wire mesh were used for this study (Martin's Cages, Nanticoke, PA, Model # H-600HR). The dimensions of these units were 18" W x 11" D x 24" H. The towers each sat in a deep plastic pan lined with Teklan soft cob bedding (7087c) which served as the bottom floor. The towers also included a central floor, along with smaller lower and upper landings. A sign-tracking apparatus was placed in each of the towers. (see Appendix A). These devices consisted of a retractable sipper (Med Associates Inc., St. Albans, Vt, ENV-252M) and a pellet dispenser, (Gerbrands Co.) located on the upper landing of each tower. A food tray was secured to the middle of the upper landing wall and was recessed so that the front was flush with the tower wall. The food tray, into which sugar pellets (Noyes, 45 mg) were delivered, was located immediately to the right of a hole in the wall through which the sipper could be pushed through for sign tracking. Head pokes into the food tray were measured by photosensors (Med Associates Inc., ENV-303HD), and licks to the sipper bottle were measured by a lickometer controller (Med Associates Inc, ENV-250B). A speaker attached to the same mesh wall as the other devices (far right side) was used to emit a white noise discriminative stimulus indicating the start of a sign-tracking session. The top half of the front of each tower was covered with a black sheet in order to limit light and visual distractions from the room. All programmed schedules were controlled with Med Associates interface equipment and written using Med PC notation.

Procedure

A Summary of the procedure timeline is shown in Table 1.

Adaptation. After being assigned to one of two groups and being placed into their respective home tower or cage, rats were handled daily for about two weeks and given time to become acquainted with their new environments and cage mates. During this period, they were permitted ad libitum access to food and water. In the standard cages, the food and 2 water bottles were located on the top of the cage. In the towers, the food was located on the bottom floor, and two water bottles were secured side by side to the wall of the middle floor. All rats were provided initial exposure to sucrose pellets during this phase. The standard group received 10 pellets and the environmental enrichment group received 20 pellets every other day scattered on the bedding.

After three days of adaptation, an assortment of objects (chew toys, toilet paper rolls, PVC pipes, etc.) were introduced into the towers for the enrichment condition. The objects continued to be changed out about two times per week for the remainder of the experiment. The Standard Housing groups were not given access to any of these objects.

Pre-exposure to EtOH and Introduction of Two-Bottle Choice tests. After the adaptation phase and before sign-tracking procedures began (13 days after arrival to the lab), all rats were familiarized with ethanol (EtOH), and initial EtOH consumption and preference were measured by two-bottle choice tests. For this procedure, water and 2% ethanol solution were placed into the side by side bottles in each cage/tower, and the amount consumed of each was measured to determine any initial preference for one over the other. This was repeated for 10 days, with the ethanol concentration gradually increasing by 1% increments until 5% ethanol was reached. The left/right position of the water bottle and EtOH bottle alternated every other day to avoid the development of a side preference. In the standard cages, the bottles were placed side by side next to the food on the top of the cage. The bottles in the towers were placed adjacent to one another on the middle floor. The intakes of EtOH and water for each cage/tower were measured daily in grams, with the exception of Sundays. Because no study personnel monitored the rats on Sundays, weekend intakes were recorded as averages. Since overnight two-bottle choice tests did not allow us to distinguish which rats were drinking, each rat was tested individually for each concentration after 2%. Alcohol was not available in the home cages the night before individual testing so that the rats that had developed a preference for it would be motivated to drink. These individual two-bottle tests lasted 20 minutes and used the current EtOH concentration along with water. For these individual tests, the rats were tested in four individual stainless-steel cages in a separate room from their home cages. Four rats were tested at a time, one in each cage. We carried both overnight and individual two-bottle choice tests into the sign tracking phase of our experiment, to examine changes in alcohol preference throughout the next phase.

The pre-exposure phase lasted a total of 20 days. Therefore, at the conclusion of this phase, the tower rats had experienced roughly five weeks of environmental enrichment prior to sign tracking.

Sign-Tracking Procedure. After EtOH pre-exposure, all rats underwent sign tracking in a tower. Rats in the environmental enrichment group were trained in their respective towers. The standard-housed rats were randomly assigned to one of the towers and tested only in that tower. A plastic partition blocked the rats from accessing the lower levels of the towers during sign tracking. All other rats were removed from the towers during these sessions and temporarily placed in standard cages. All rats were given two days of adaptation during the third week of EtOH preexposure with ten sucrose pellets in the food tray and the white noise turned on. All rats consumed the sugar pellets within ten minutes, so no food deprivation was necessary during adaptation.

Sign tracking for each rat took place roughly every other day, since it was not possible to run all 16 rats in the same day. Sign-tracking sessions took place in the mornings, Monday through Saturday. Whichever group was tested on Saturday was tested again on Monday to ensure balanced testing schedules.

The start of each session was indicated by a white noise discriminative stimulus. After 60 seconds, the sipper bottle, containing 5% EtOH , would be pushed into the apparatus by a pre-programmed mechanical arm so that it was made accessible to the rat being tested. After ten seconds, the sipper was be retracted so that it was no longer accessible, and a sugar pellet was delivered into the food tray immediately after. A single session consisted of 25 bottle-pellet pairings, separated by a 60-second inter-trial interval. Each session lasted roughly 30 minutes. The bottle containing the alcohol solution was weighed before and after each session, and weights were recorded. Upon completion, the rats were placed back into their home cages (standard group) or in a temporary holding cage (enriched Group) while the rest of the sessions were conducted. Once sessions for the day concluded, all enriched group rats were returned to their towers. Since sign-tracking behavior takes time to develop (Srey et al, 2015), these sessions were run daily for six weeks, or 18 sessions per rat.

We conducted periodic individual two-bottle choice tests during the sign-tracking phase in addition to regular overnight tests. We conducted one early on in sign tracking (week 1) and another later on (week 5) under a free feeding schedule to assess whether EtOH preference changed with increased sign tracking experience. In an attempt to induce greater intake levels during individual two-bottle tests, we implemented a post prandial feeding schedule on weeks 2 and 5 of sign tracking prior to two-bottle choice testing. For these tests, rats were food restricted overnight and then allowed to feed without access to water for thirty minutes prior to testing. Therefore, we analyzed a total of four individual two-bottle choice tests during sign tracking.

Dependent Measures Sign- and goal-tracking behavior. Sign-tracking behavior was measured by taking the mean number of licks to the EtOH sipper and the volume of solution consumed during sign tracking sessions. Goal-tracking behavior was characterized by the number of head pokes into the pellet tray during the bottle (CS) presentation vs. during an equivalent period of time (10sec) before bottle presentation (pre-CS). If the rat head poked more during the CS than the pre-CS period, it was considered to be goal tracking.

EtOH intake and preference. The home-cage two-bottle preference tests were used to measure how much EtOH the rats in each cage/tower collectively drank before, during, and after the sign-tracking phase when water was also available. These data would essentially tell us how the pattern of preference for EtOH changed over time. To compare the two groups (enriched vs.

standard housed), the average individual absolute intake was estimated for each test. To do this, we divided the absolute intake (amount of EtOH or water consumed in grams) by the number of rats in the cage (4 for the enriched condition, 2 for the standard condition). In addition to the absolute solution intake, the percent of EtOH consumed relative to water was calculated with the formula (EtOH consumed/ EtOH + Water consumed) * 100, to represent preference. We also conducted individual two-bottle tests in separate test cages for which the absolute intake and the percent of EtOH consumed was calculated for each rat individually.

Data Analysis

Our statistical analyses consisted of a series of mixed ANOVAs. For sign tracking behavior, or "wanting," we looked for main effects of Housing and 2-day Blocks on average licks to the ethanol sipper and total volume of ethanol solution consumed. A significant difference between housing conditions in mean licks per session and amount of ethanol consumed would indicate faster acquisition of sign tracking for one group, while a main effect of Blocks would indicate an increase or decrease of sign tracking over time.

Mean number of head pokes per session was the primary dependent variable used to describe goal-tracking behavior. A mixed ANOVA was conducted to determine whether a change in mean number of head pokes per session existed as the result of an interaction between period, number of days, and rearing/housing condition. A main effect of period, or significantly more head pokes during the CS than during the pre-CS period would indicate conditioned goal-tracking behavior. It is also important to keep in mind that goal tracking might decrease as sign tracking increases, reflected in a period by day interaction. We would expect to see an interaction between all three independent variables (condition, period, and days) if the emergence of goal-tracking behavior and its gradual reduction was influenced by housing condition.

Liking was measured by ethanol preference (%) and total ethanol intake (g) during the 24-hour two-bottle choice procedures in the home cage and the 20-minute two-bottle tests outside of the home cage. No inferential statistics were conducted on the data collected from the home cage tests, since for each test there were four measures for the standard group (1 per cage) and only two for the enriched group (1 per tower).

Mixed ANOVAs were conducted for ethanol preference and total ethanol intake recorded during the tests that occurred *outside* of the home cages to find whether changes in these variables were results of an interaction between the rearing/housing condition and the time of the two-bottle test. A main effect of time would indicate a change in preference as a result of repeated experience with alcohol, and a Condition x Time interaction would indicate a differential effect of housing condition on preference over time.

Results

Acquisition

Sign tracking. Sign tracking acquisition for each group can be seen in Figure 1. The dependent variable used to indicate sign-tracking behavior was average number of licks to the ethanol sipper. We confirmed that the average number of licks during sign tracking did not differ between the two towers, F (1,14)=1.132, p=0.305, η_p^2 =.029. A 2 x 9 (Housing [enrichment, standard] x Blocks [1-9] ANOVA produced a significant main effect of group, with the environmental enrichment group exhibiting more total licks to the ethanol sipper during sign tracking sessions than the standard group, F (1,14)= 10.40, p = 0.006, η_p^2=0.426. There was a simple main effect of blocks ,(1,14=17.37, p <.001, η^2_p =.55, and follow-up t-tests revealed that rats produced more licks to the sipper during blocks 3-7 than during the first two blocks (*ps*<.01). Furthermore, we found a significant Housing x Blocks interaction, $F(1, 14)=2.451$, $p=0.018$, $\eta^2_p=0.045$. Post-hoc analysis revealed that the enrichment group had a higher rate of licks than did the standard group during blocks 3, 4, 5, 7, and 9 (*ps* < .05).

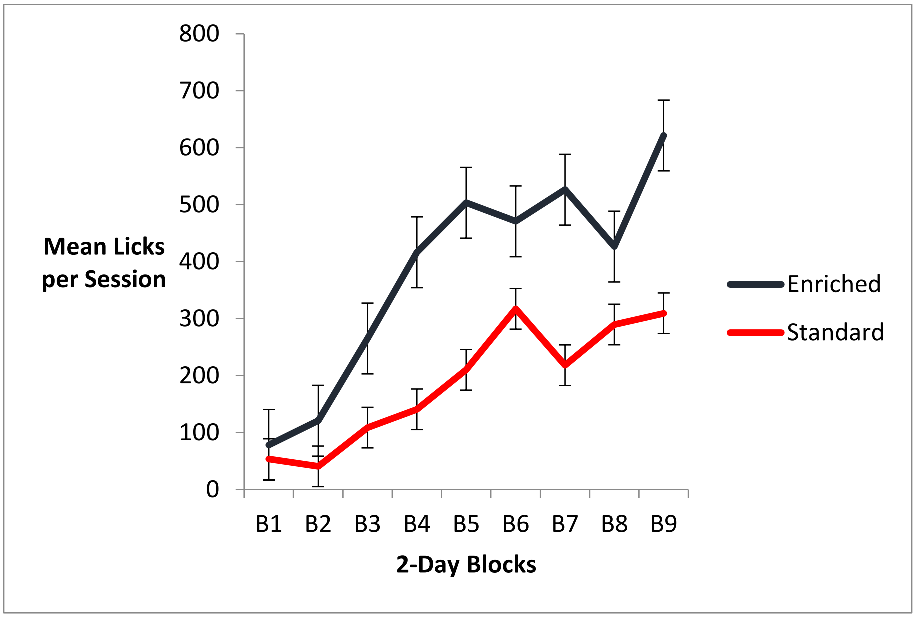
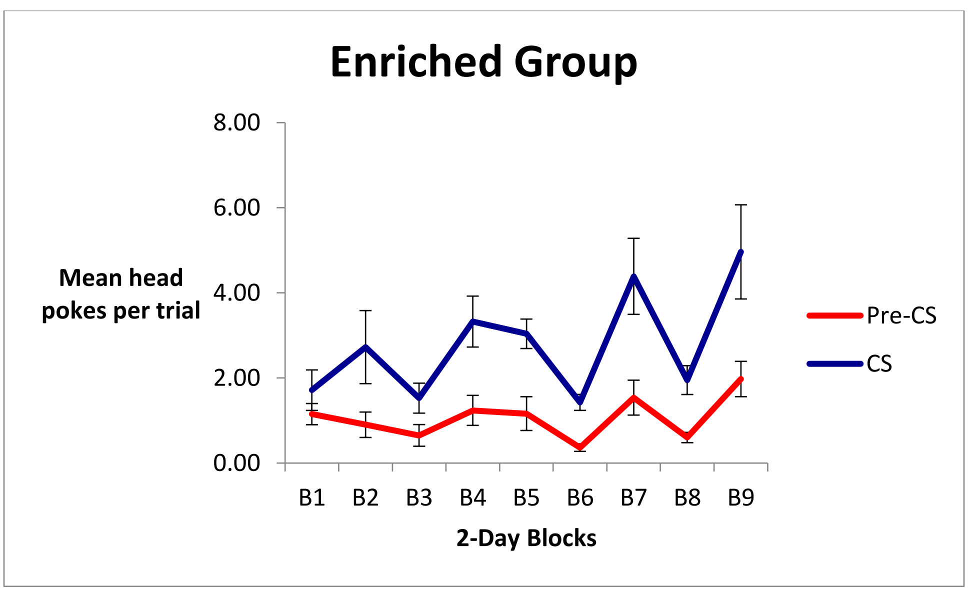


Figure 1. Sign tracking acquisition. Each block represents a two-day average of licks to the ethanol sipper during sign tracking session.

Goal tracking. Goal-tracking behavior was measured by the average number of headpokes into the pellet delivery site during the pre-CS and CS periods. We did observe a statistically significant difference in the number of headpokes in each of the towers, $F(1,14)= 8.88$, $p= 0.01$, $\eta^2_p=0.39$, where the number of headpokes was greater overall in Tower 1 than in Tower 2. This result may be linked to a peak on Day 9 (within 2-day Block 5), in which headpokes in Tower 1 were remarkably high for an inexplicable reason. Both housing groups displayed goal-tracking behavior, as evidenced by headpokes recorded during the pre-CS and CS periods. We conducted a 2 x 2 x 9 mixed ANOVA (Housing [enrichment , standard] x Period [preCS, CS], x Blocks [1-9]) to investigate group differences and trends in acquisition. We found a significant Period x

Blocks interaction, indicating that the number of headpokes during the CS period versus the pre-CS period increased over training blocks, F (1,14)=8.17, p<.001, η^2_p =0.053 This interaction reflects the acquisition of conditioned headpoking behavior (i.e., goal tracking) . There was no statistically significant main effect of group on mean headpokes per trial, F (1,14)=0.505, p=0.489, η^2_p =0.039, however we did observe an obvious difference in trends between the groups. We see a general increase in headpokes over time for the enrichment group, as CS headpokes during blocks 7 and 9 are significantly greater than headpokes during block 1 (p=0.006, p=0.009). However, dramatic drops in headpokes during blocks 6 and 8 make interpretation difficult. We suppose there may have been equipment troubles on those days or an event in the lab that impacted performance. On the other hand, mean headpokes for the standard group steadily decreased during the pre-CS period and increased during the CS period, reflecting greater conditioned goal tracking than the enrichment group. This difference is reflected in a significant 3-way interaction between Housing, Blocks, and Period, F(1,14)=3.21, p<.05, η^2_p =.19. Goal tracking acquisition for each group can be seen in Figure 2.



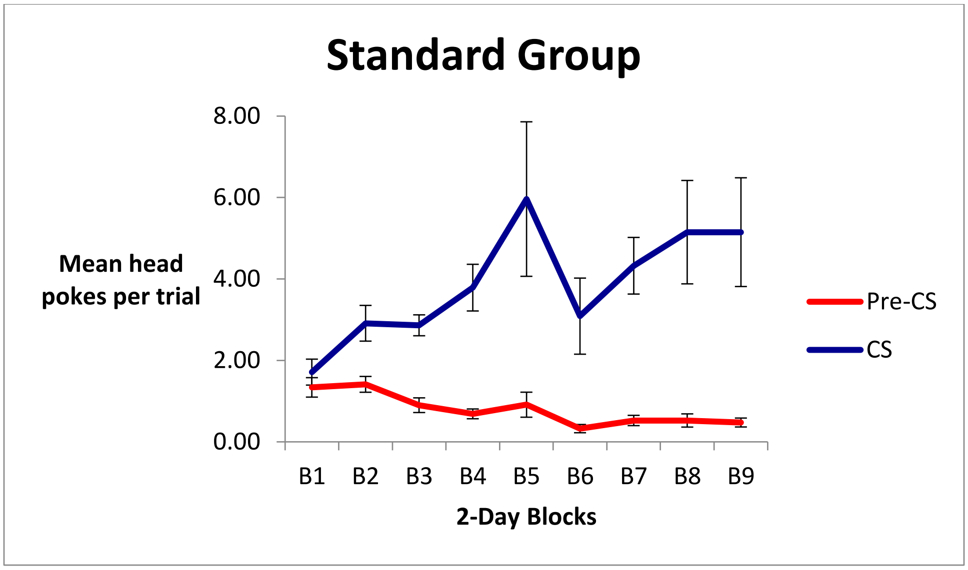


Figure 2. Sign tracking acquisition. Each block represents a two-day average of licks to the ethanol sipper during sign tracking session.

Correlations. We were interested in seeing whether higher sign tracking was associated with lower goal tracking, so we conducted correlations between lick rates and head pokes. Correlations for the enriched and standard groups combined for the 9 blocks of training can be seen in Figure 3. Seven of the nine blocks yielded negative correlations, however none approached statistical significance. Block 4 was an anomaly with rats displaying a statistically significant positive correlation, $r=0.764$, $p<.001$. Mean lick rate and headpoke correlation for each group separately can be seen in Figure 4. Although the sample size is reduced by half, we examined the correlations of the two housing groups separately to determine if there was any indication that the correlation between the two measures was influenced by the housing condition. The enriched group showed more negative correlations early in training, otherwise no clear group difference was apparent.

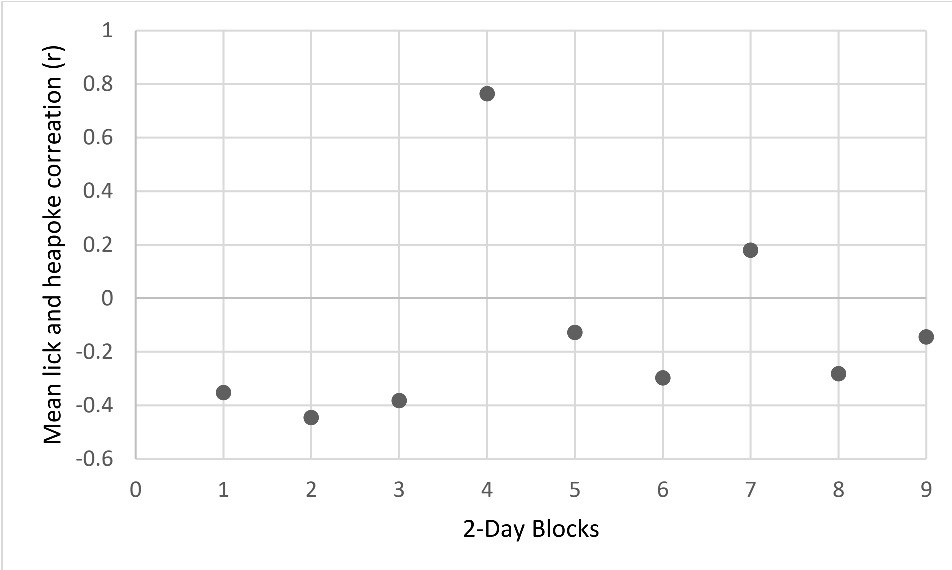
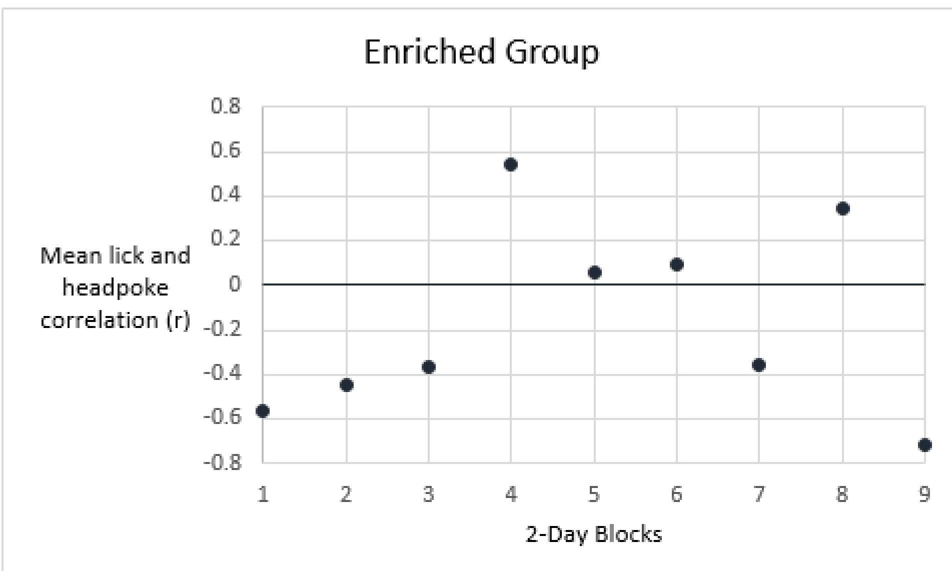


Figure 3. Pearson correlations between average lick rates and headpokes for the standard and enriched groups combined.



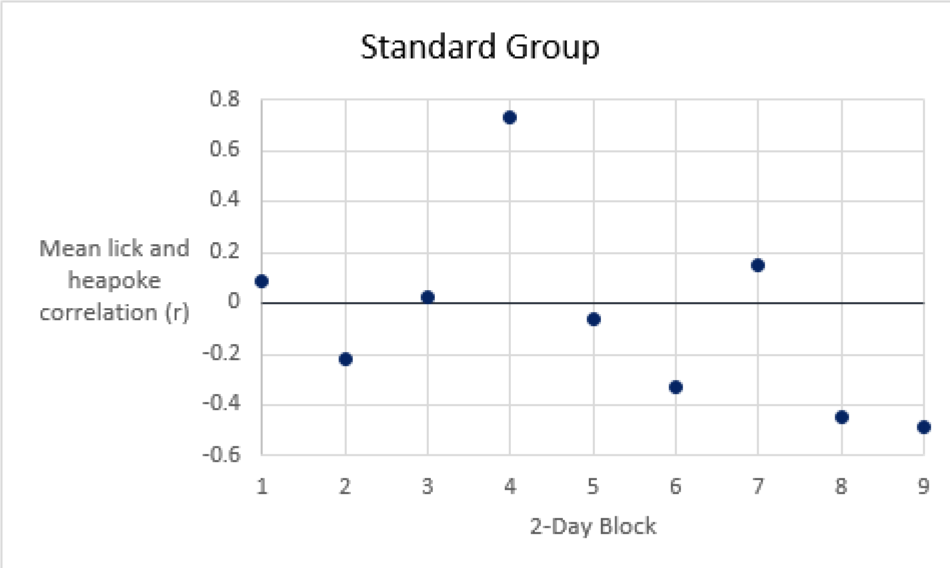


Figure 4. Correlations between average lick rate and headpokes for the enriched and standard housing groups. Correlations are represented as Pearson's r.

Extended Sign Tracking Training. Similar to a prior experiment in this laboratory, we observed during the last few days of acquisition a pattern of responding in which the number of licks started out high in the early trials of a session and decreased in the later trials for the environmental enrichment group. This trend was not apparent for the standard group. To further evaluate this effect, we extended training for 9 additional sessions. The patterns of within-session lick rates for all 19 days of training can be seen in Figure 5. To evaluate group differences in these within-session declines in lick rates (i.e., sign tracking) the 25 trials per day were recalculated as 5-trial block means and we analyzed the last 12 days (days 7-18) with a 2 x 5 x 12 mixed ANOVA (Housing [enrichment, standard] x Trial Blocks [1-5], x Days [7-18]). As expected, lick rates averaged over days and blocks were significantly greater for the enriched group than for the standard group, $F(1,14)=10.10$, $p<.01$, $\eta^2_p=.42$. We found a significant main effect of Trial Blocks, $F(1,14)=8.11$, $p<.001$, $\eta^2_p=.14$, which confirmed that rats did, in fact, display greater lick rates during earlier blocks (1,2,3) than on later blocks (5) (*p*s<.05). A significant Housing x Trial Blocks interaction revealed that this finding only applied to the enriched group, as standard rats displayed even lick rates throughout all five of the trial blocks. To further investigate group differences, we analyzed each group separately. We found a significant Days x Blocks interaction for the enriched group [$F(7)=3.25$, $p<.001$, $\eta^2_p = .317$] but not for the standard group [$F(7)=1.17$, $p=.22$, $\eta^2_p = .03$]. Furthermore, we saw a significant three-way interaction between housing, trial blocks, and days, $F(1,14)=1.80$, $p < .01$, $\eta^2_p = .11$. Within session lick rates can be seen in Figure 5.

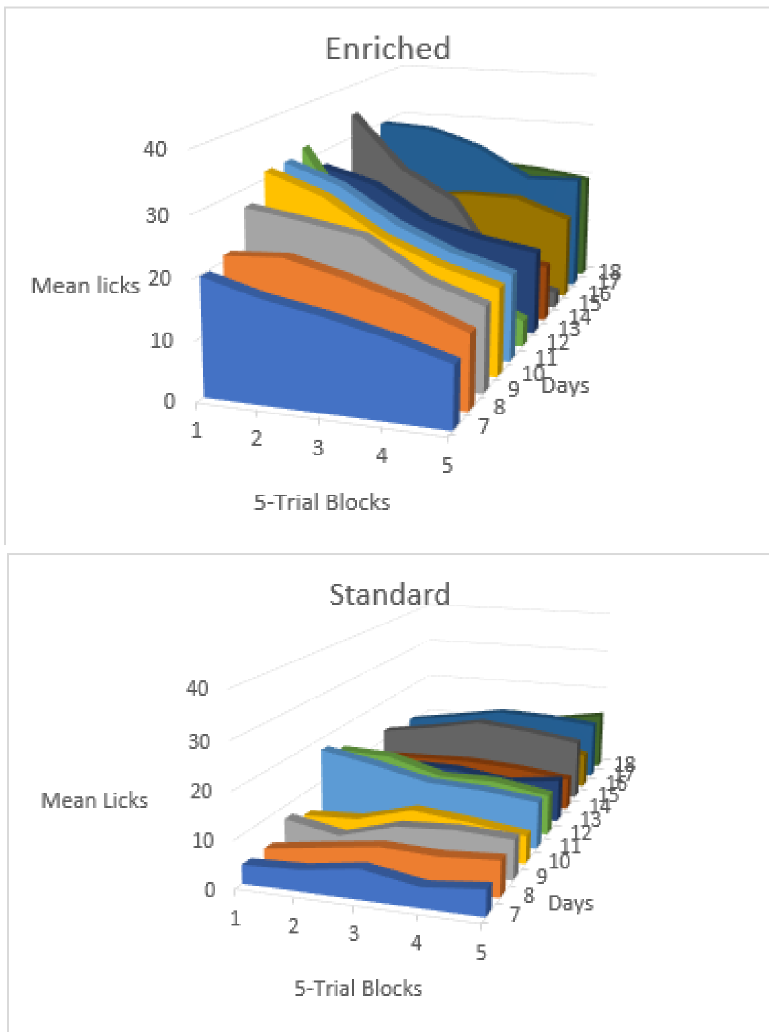


Figure 5. Within session lick rates for each group over days 7-18 of sign tracking. 25 trials have been averaged into 5-trial blocks.

Ethanol Consumption and Preference

Ethanol intake during sign tracking. For our analysis of EtOH consumption during acquisition of sign tracking sessions, we ran a 2 x 9 mixed ANOVA (Housing [enriched, standard] x 2-Day Blocks [1-9].We found a significant main effect of group, $F(1, 14)= 6.88$, $p<.05$, $\eta^2_p=.33$, with the enriched group consuming significantly more EtOH solution than the standard group, which is consistent with the pattern we found when analyzing lick rates. Additionally, we found a significant main effect of 2-day blocks, $F(1,14)=3.76$, $p<001$, $\eta^2_p=.21$. Post-hoc analysis revealed that rats drank more during later sessions (block 9) then earlier sessions (blocks 1 and 2), *p*s<.05. We did not find a significant 2-Day Blocks x Housing interaction, $F(1,14)=2.00$, $p=.11$, $\eta^2_p=.13$. EtOH intakes during sign tracking sessions can be seen in Figure 6.

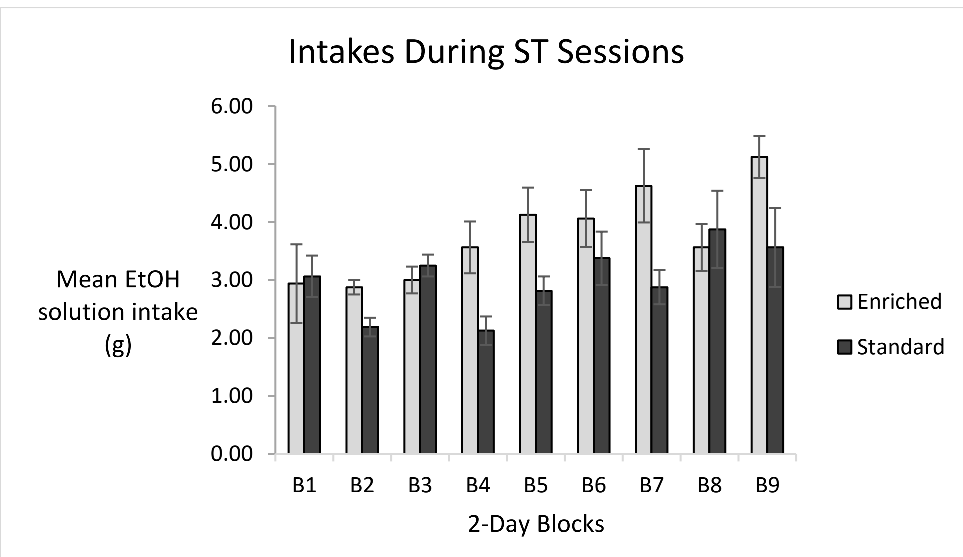


Figure 6. Average EtOH solution intakes for each group during sign tracking sessions

Group ethanol intake and preference in the home cage. Overnight two-bottle group preference tests were conducted during the pre-exposure phase as well as at the end of each week of sign tracking (except for week 1) by placing one bottle of ethanol and one bottle of water on each tower (Enriched group) or home cage (Standard Group). Because the intake of all rats in a tower or cage were taken rather than the individual rats' inferential statistics could not be done given the small n. We calculated the average amounts of ethanol consumed during overnight two-bottle choice tests for each group during each week of sign tracking and divided by 2 (for standard house) and 4 (for towers) to find the average ethanol intake per rat. Whereas the average intake remained low for enriched rats throughout the five weeks and remained relatively stable, intake for the standard rats increased steadily from before sign tracking began to after the fourth week of sign tracking. We saw a drop in ethanol intake during the 2-bottle test following the fifth week of sign tracking, but in general intake tended to increase with more sign tracking experience.

We also calculated the preference for ethanol over water for each week of two-bottle preference tests. We found that ethanol preference roughly followed the pattern of ethanol intake. Preference for ethanol in the enriched group decreased after week two of sign tracking, which was the opposite effect as what we saw in the standard group. For the standard rats, ethanol preference increased each week from before sign tracking to after week 4 of sign tracking, then dropped on week 5, reflecting the trend we observed when looking at absolute intake. Average absolute intake and average preference during overnight two-bottle tests can be seen in figures 7 and 8, respectively.

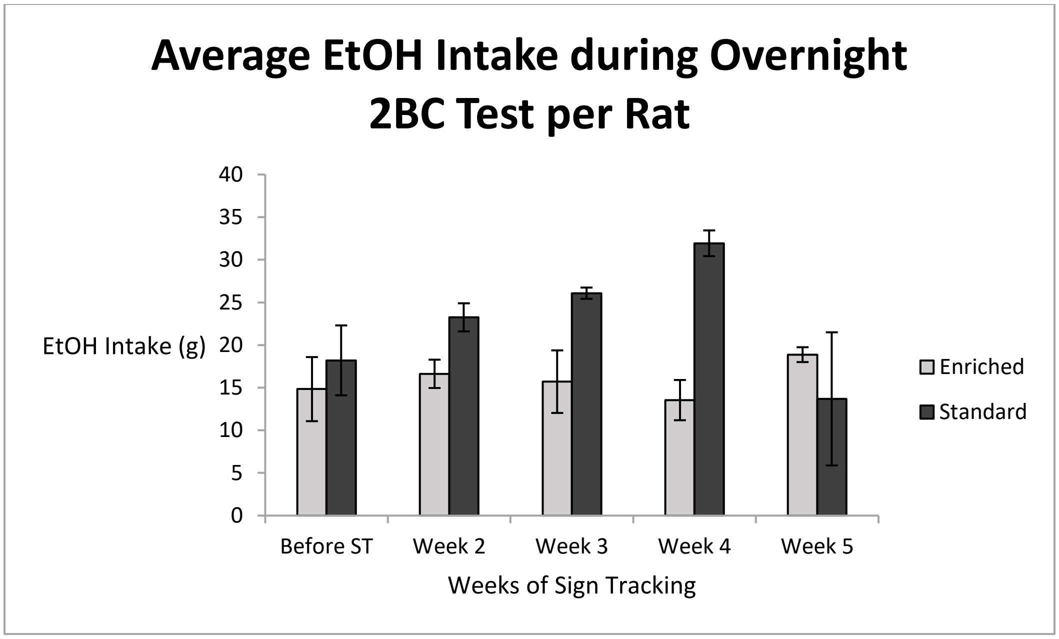


Figure 7. Average EtOH intake per rat in grams during overnight two-bottle preference tests conducted within the home cage before sign tracking and after each week of sign tracking

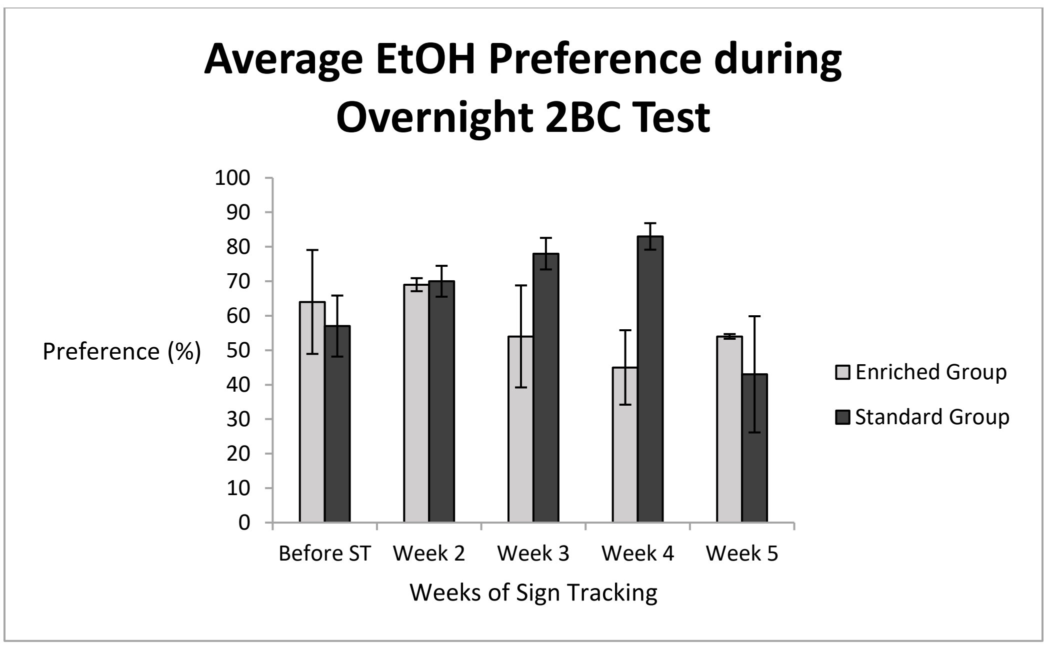


Figure 8. EtOH preference during overnight home cage two-bottle choice tests for each group before sign tracking and after each week of sign tracking

Short-term two-bottle tests outside of the home environment. We used two different measures to analyze "liking" of ethanol outside of the home environment during 20-minute individual two-bottle preference tests. The first was absolute EtOH intake during the test, and the second was the percent of EtOH solution consumed out of the total volume of liquid consumed (preference).

Pre-Exposure. For the pre-exposure period (weeks leading up to the sign tracking phase), we ran a 2 x 3 ANOVA (Group [enriched, standard] x Concentration [3%, 4%, 5%]. There was a main effect of Concentration on average ethanol intake during 20-minute individual two-bottle choice tests, $F(1,14)=8.77$, $p<0.001$, $\eta^2_p=0.31$. Rats consumed significantly more ethanol at 4% and 5% than at 3%. We also found a significant main effect of Group, $F(1,14)=6.750$, $p<0.05$, $\eta^2_p=0.32$, and a significant Concentration x Group interaction, $F(1,14)=4.92$, $p<0.05$, $\eta^2_p=0.08$. Additional post-hoc tests indicated that the enriched group consumed significantly more ethanol than the standard group at 5% concentration $p<0.05$). Individual 2-bottle preference test data can be seen in Figure 9.

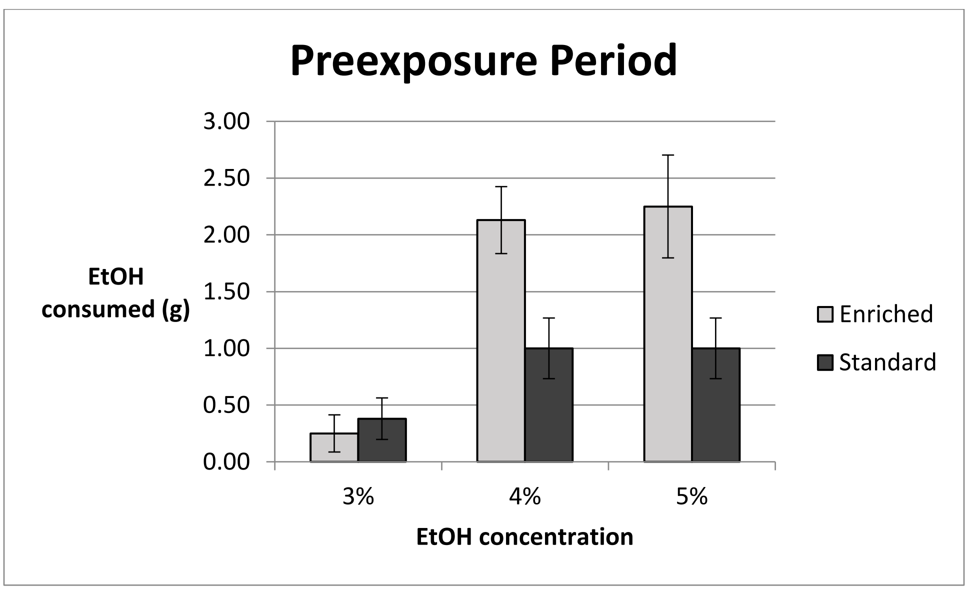


Figure 9. Twenty-minute individual 2-bottle preference tests conducted outside of the home towers/cages during the pre-exposure period

During Sign-Tracking. We used a 2 (Housing [enriched, standard]) x 2 (Weeks of Sign Tracking [week 1, week 4]) ANOVA to analyze group differences in absolute ethanol intake during individual two bottle tests at different points of the sign tracking phase. We found a significant main effect of Housing condition, $F(1,14)=10.33$, $p<0.01$, $\eta^2_p=0.43$ and a significant Weeks of Sign Tracking x Housing interaction, $F(1,14)=9.96$, $p<0.01$, $\eta^2_p=0.18$. Enriched rats drank significantly more ethanol than standard rats on week 1 ($p<.001$), but not week 5 of sign tracking. Furthermore, enriched rats drank significantly more ethanol on week 1 than they did on week 5 ($p=.01$). When comparing preferences on the other hand, we did not find differences between the enriched group ($\bar{x}_{\text{Week 1}}=63.18$, $\bar{x}_{\text{Week 5}}=60.63$) and the standard group ($\bar{x}_{\text{Week 1}}=64.59$, $\bar{x}_{\text{Week 5}}=48.03$), $F(1,14)=.25$, $p=.48$, $\eta^2_p=.02$. Furthermore, we did not find evidence of preference changing over time, $F(1,14)=.54$, $p=.627$, $\eta^2_p=.01$.

Despite the group differences in ethanol intake described above the absolute intakes were generally low, we tried to increase overall drinking during the two-bottle preference test by giving the rats food only (no water) to induce post-prandial drinking, once following week two of sign tracking and again at the end of week five. Rats were food deprived overnight, then provided with food (but no water) for thirty minutes prior to the preference tests, this was expected to induce rats to drink more after taking a meal with no available water. When analyzing group differences with a 2x2 mixed ANOVA (Housing [enriched, standard] x Weeks of Sign Tracking [2, 5], we did not find a difference between the enriched group (, $\bar{x}_{\text{Week 2}}=3.75$, $\bar{x}_{\text{Week 5}}=4.00$) and the standard group ($\bar{x}_{\text{Week 2}}=3.00$, $\bar{x}_{\text{Week 5}}=2.25$ for average absolute intake, $F(1,14)=3.27$, $p=.09$, $\eta^2_p=.19$. We also saw no significant change in intake over time from week 2 to week 5, $F(1,14)=.09$, $p=.77$, $\eta^2_p=.01$ and no Housing x Weeks of Sign Tracking interaction, $F(1,14)=.34$, $p=.57$, $\eta^2_p=.02$. We found no significant difference between housing groups when analyzing EtOH preference either under post prandial testing, $F(1,14)=.01$, $p=.94$, $\eta^2_p <.01$ and no Housing x Weeks of sign tracking interaction , $F(1,14)=.93$, $p=.35$, $\eta^2_p =.06$. EtOH intake under free feeding and post prandial testing can be seen in Figure 10, and preference can be seen in Figure 11.

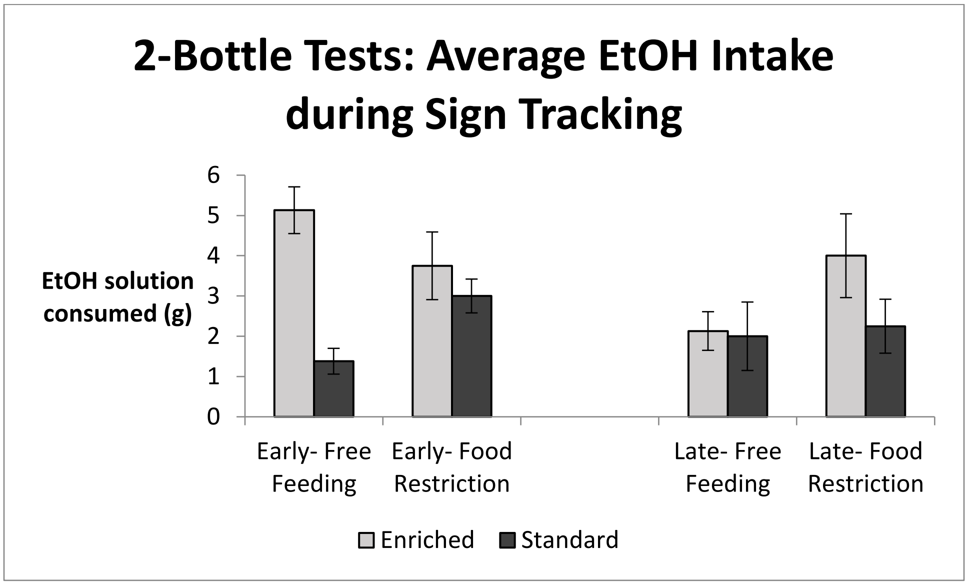


Figure 10. Average absolute ethanol intake early (1 week) and late (5 weeks) into sign tracking.

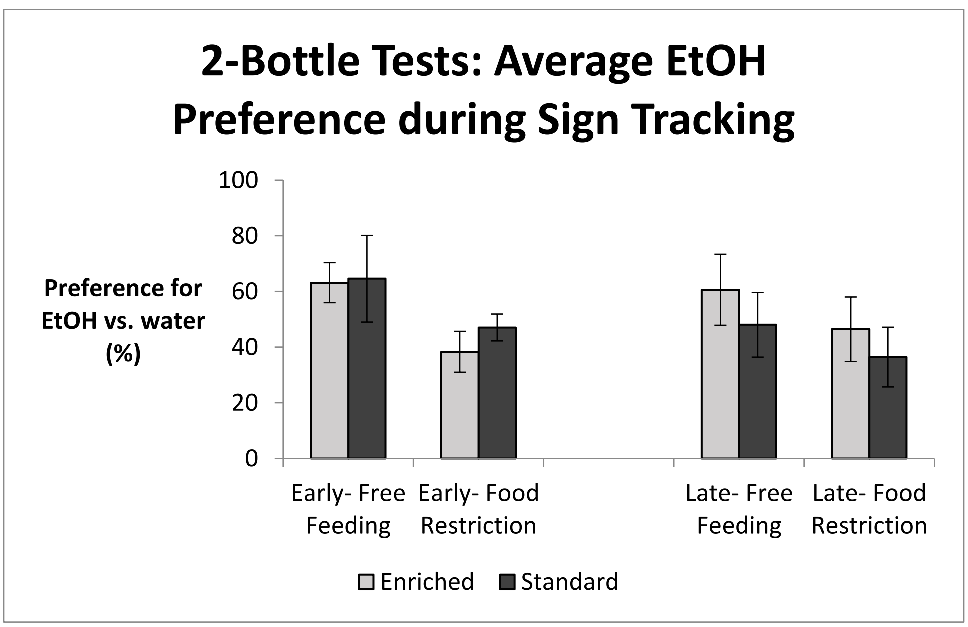


Figure 11. Preference for EtOH solution over water early (1 week) and late (5 weeks) into sign tracking

Discussion

Testing within a more natural environment did not appear to preclude the acquisition or maintenance of sign tracking. Furthermore, the presence of significant differences in sign tracking behavior between the enrichment and standard housing groups confirmed our suspicion of the environment as an influencing factor. We found that, overall, the enrichment group sign tracked at a stronger rate, while the standard group displayed stronger conditioned goal tracking.. Detailed explanations of our findings are presented in the following subsections.

Sign Tracking. We found evidence of robust sign tracking behavior, as demonstrated by the average recorded licks to the ethanol solution bottle during testing sessions. Lick rates for both housing groups increased significantly after the first two blocks (4 days), indicating the learned association of the CS/US pairings over time. The enriched group displayed stronger acquisition of sign-tracking behavior, evidenced by greater lick rates early on (blocks 3-5) and continued to display greater lick rates than the standard group throughout the 9 blocks (18 sessions). When looking at later sessions, we see that average lick rates for the standard group appeared to be approaching an asymptote, while lick rates for the enriched group had not yet stabilized and reached a peak on block 9. Because of the apparent differences in the magnitude and patterns of lick rates between the two housing groups, our findings support the hypothesis that environmental enrichment has some influence on sign-tracking behavior.

As mentioned in the introduction, studies investigating environmental enrichment and sign tracking have yielded contradicting results. In a study conducted by Beckmann and Bardo (2012), rats raised in an enriched environment tended to goal track more while rats raised in isolation displayed primarily sign-tracking tendencies during sessions, supporting the idea that environmental enrichment may reduce the propensity to attribute incentive salience to reward-associated cues. The results are opposite of what we found in our experiment. Numerous methodological differences could help explain conflicting findings, one being the nature of each CS. Beckmann and Bardo used a retractable lever CS, rather than a bottle containing ethanol solution as in our study. While the incentive sensitization theory suggests that any CS paired with a reward US may acquire some incentive value of its own, the ethanol solution is rewarding in itself by its pharmacological effects. It may be possible that the enrichment rats are more sensitive than the standard rats to the rewarding effects of the ethanol during intermittent exposure but not necessarily more likely to attribute incentive salience to reward associated cues. However, the volume of solution consumed during each session renders this explanation somewhat unlikely. By block 5 (days 9 & 10), rats in the enriched group were only drinking an average of about 4 grams of solution per session, which may not be enough to induce pharmacologically relevant effects. Furthermore, in an unpublished master's thesis conducted at Seton Hall University, greater sign tracking was observed in environmental enrichment rats than standard rats during acquisition with a water bottle CS, indicating that pharmacological effects of the CS may not be a relevant factor in group differences (Casachahua, 2011).

Another key procedural difference between the experiments is the amount of sessions carried out for each animal. Beckmann and Bardo ran a total of 5 sessions, while we extended training to 18 sessions. With sufficient training, it is possible for primarily goal tracking animals to transition to primarily sign trackers (Srey et al, 2015). The CS and US paired together repeatedly results in the learned association over time that is expressed in behavior change that eventually reaches an asymptote. Sign tracking for our animals increased significantly after block 2 (days 3 & 4), indicating that a strong association (or the behavioral manifestation of this association) may not have been formed in earlier sessions. Furthermore, we did not find a significant difference in lick rates between housing groups until block 3. If the rats in the Beckmann and Bardo study were to follow a similar trend, they may not have had a chance to acquire strong sign-tracking behavior within the 5 sessions allotted or transition from goal tracking to sign tracking.

Finally, different “control” groups could partially account for contradicting results. We compared environmental enrichment rats housed in groups of 4 with those paired in standard laboratory cages as opposed to in isolation. Greater sign-tracking behavior exhibited by rats housed in isolation (Beckmann & Bardo, 2012) could have been driven by a factor such as increased stress, since rats are known to be social animals. Perhaps stress due to isolation may increase an animal’s propensity to attribute incentive salience to a reward cue.

In the aforementioned Seton Hall University Master’s thesis, environmental enrichment rats sign tracked an ethanol bottle CS (after initial acquisition with a water bottle) at a stronger rate than did rats housed in paired in standard cages (Casachahua, 2011). These findings, in agreement with ours, contradict the prevailing idea that environmental enrichment may serve as a protective factor against compulsive- or abuse-like behavior. In order to comprehend our results, it may be worthwhile to analyze sign tracking from different approaches.

One perspective our findings support is that sign tracking may not be as automatic and inflexible a response as has been reported by some (Ahrens et al, 2016; Tomie & Sharma, 2013). In fact, the environmental enrichment rats showed adaptability to the procedure as evidenced by patterns of within-session habituation. Persistent high lick rates to mark the beginning of each session indicated acquired salience of the ethanol sipper CS, while decreasing lick rates throughout each session portrayed an ability to adjust the behavior. This trend was more pronounced in the environmental enrichment group, while their standard-housed counterparts exhibited more constant lick rates throughout the duration of each session. These group differences suggest that despite greater overall lick rates, the enrichment group may have exerted greater control and behavioral flexibility over the conditioned response.

Furthermore, if sign tracking became a persistent reflexive response, we would expect to see strong negative correlations between sign tracking and goal tracking, as goal-tracking subsided and sign tracking dominated. Instead we see weak, statistically insignificant negative correlations, indicating a mixture of sign-tracking and goal-tracking activity. Furthermore, when looking at conditioned goal tracking for the enrichment group, headpokes during the CS period do not subside over time as sign tracking increases but even appear to move in a general increasing direction, suggesting that rats may have been switching back and forth between sign tracking and goal tracking during CS presentations.

Despite the examples of flexibility that we found in our sign tracking animals, much of the literature continues to portray sign tracking as an inflexible response. Several experiments have produced evidence that sign tracking is less sensitive than goal tracking to reward devaluation manipulations (Morrison et al, 2015; Ahrens et al, 2016). Interestingly, however, one recent experiment demonstrated that rats classified as sign trackers were insensitive to satiety devaluation after limited training but became sensitive following extended training (Keefer et al, 2020). While the current literature seems to be in agreement that sign tracking is less sensitive to experimental manipulation than goal tracking, it may be worth exploring possible flexibility in sign-tracking behavior to help determine the appropriateness of labeling it as a truly compulsive-like behavior.

Another popular belief is that sign tracking is a maladaptive behavior that provides no direct benefit to the animal. However, Timberlake’s behavior systems theory has provided a compelling argument that this type of “misbehavior” may be an embodiment of a set of natural appetitive behaviors evoked in the presence of a food reward (Timberlake,1984). Timberlake held that learning, even as observed within the confines of a laboratory setting, should be studied “in the context of natural problems, stimuli, and behavior,” so that any action performed by an animal may be understood as a component of a pre-existing behavior system that guides such action (Timberlake, 1984). This shift to an ecological perspective in analyzing behavior, influenced heavily by Timberlake, has been upheld as a more comprehensive approach to studying animal behavior (Cabrera et al, 2019).

Timberlake et al (1982) used Pavlovian pairings of a rolling ball bearing and a food pellet to demonstrate that rats tended to grab, chew, release, and recover the ball bearing in anticipation of obtaining the food pellet. The behavior system approach highlights that these actions closely mimic those performed by rats in a more natural environment in order to obtain a food source. Timberlake further states that an animal’s interaction with a stimulus depends on that stimulus’s resemblance to a natural cue or situation. The ball bearing paired with a food reward evokes predatory behaviors, whereas presenting another rat along with the food pellet produces social feeding behaviors (Timberlake & Grant, 1975). The predictive stimuli do not act as substitutes for the reward, but rather induce the appropriate appetitive behavior related to the reward. In our experiment, much of the rats’ actions directed toward the ethanol sipper consisted of pawing, sniffing, and chewing. In fact, while interactions with the sipper were high, the total volume of ethanol consumed remained surprisingly low throughout the sessions, indicating the occurrence of behaviors other than drinking or deliberate inefficacious licking. If the behavior systems theory holds true, one might argue that interacting with the ethanol sipper is a natural response to an affordance perceived by the animal, and that environmental enrichment might evoke more natural appetitive behaviors directed at perceived affordances.

Goal tracking. Goal tracking activity for each group was measured by the average number of head pokes into the sugar pellet delivery site. Goal tracking occurs when head pokes during the CS period significantly outnumber head pokes during the pre-CS period. We found evidence of stronger conditioned goal tracking for the standard group than for the enriched group. The goal tracking graph for the standard group (Fig 2) depicts a growing gap between CS and pre-CS headpokes, such that pre-CS headpoking activity moves in a generally decreasing direction, while the average number of headpokes during the CS period move in an upward direction. This trend indicates that the animals have successfully used the CS to predict the availability of the reward to a greater extent than when there is no cue present. Goal tracking was also evident in the enrichment group, though not as strong or consistent. Interestingly, CS headpokes did not appear to decrease over time for either group as sign tracking was acquired. Furthermore, we ran correlations and found no significant negative correlations between head pokes and licks, meaning that greater sign tracking was not associated with lower goal tracking. Therefore, it is likely that animals were displaying both sign-tracking and goal-tracking behaviors during the CS period, possibly switching back and forth between the ethanol sipper and the sugar pellet delivery site. If this is true, concept of sign tracking as an "inflexible" response is further diminished. Were sign tracking as involuntary as has been claimed, we would have expected goal tracking to give way to sign tracking over time, rather than compete with it.   However, we this was not the case for either group, even the enrichment animals who demonstrated stronger acquisition of sign tracking.

Two-Bottle Choice Tests. Whereas sign tracking was used as a measure of "wanting," periodic two-bottle preference tests conducted outside the home cage were implemented as measures of liking, or preference for the ethanol solution. We were curious as to whether the acquisition of sign tracking and extended sign tracking training would correspond with changes in preference and average intake levels during two-bottle tests. To test this, we conducted individual 20-minute two-bottle choice tests beginning several weeks prior to sign tracking and continued running weekly sessions through the end of the experiment.

Two-bottle choice tests were included during the pre-exposure phase of the experiment for two primary reasons: 1) so that rats were made familiar with the solution (i.e. the solution itself was not "novel" upon the beginning of sign tracking) and 2) to assess differences in baseline "liking" between the two groups. We found that during the pre-exposure period, environmental enrichment rats consumed significantly greater amounts of both 4% and 5% ethanol solution during individual two-bottle choice tests than standard housed rats which is a noteworthy observation, as the enrichment group subsequently displayed faster acquisition of sign-tracking behavior than did the standard group. This group difference was maintained following the first week of sign tracking at 5% ethanol solution, but not after extended sign tracking training. Interestingly, the enrichment rats consumed on average *less* ethanol solution on week 5 of sign tracking than on week 1, suggesting that liking for ethanol did not increase with stronger sign-tracking behavior. Furthermore, we found no group differences in ethanol consumption during two-bottle choice tests following week 5 of sign tracking. We also analyzed preference for ethanol solution versus water, which yielded no significant group differences or changes over time, although food depriving the rats overnight and running the tests post prandial on weeks 2 and 5 resulted in decreased ethanol preferences.

A lack of notable trends in individual two-bottle tests could possibly be due in part to the limited duration of each session and low volumes of liquid consumed. We supplemented these tests by also running the overnight two-bottle choice tests in home cages following each week of sign tracking, allowing rats time to consume more significant amounts of water and solution. Although we were unable to run inferential statistics for these sessions, we did observe increasing average intakes for the standard group, in contrast to the more stable volumes for the enrichment group week-to-week. Moreover, the standard group averages appeared to be greater than those of the enrichment group from the pre-exposure period through week 4 of sign tracking. Preferences showed a similar trend, increasing for the standard group over time while even slightly decreasing for the enrichment group. At a glance, liking appeared to be elevated for the standard group, but not the enriched group during sign tracking, which we did not find evidence of from the individual two-bottle tests. The observation of lower average intakes by the enrichment group during overnight tests supports the concept of environmental enrichment as a protective factor against excessive substance use and falls in line with other studies demonstrating lower drug consumption by rats in enriched settings as opposed to rats in standard settings or isolation (Alexander et al, 1981; Kulkosky et al, 1980). Again, it is important to note here that we can only discuss patterns and not inferential statistics. Furthermore, since the tests were conducted within the home cages, it is impossible to determine how much individual rats were drinking. A possible future direction could be extending the duration of individual two-bottle choice tests to allow for greater consumption and test whether there is a relationship between individual rats' lick rates and preference for ethanol solution. This structure could also inform us whether the group differences we inferred from the overnight tests emerge in individual tests with extended durations as well as the statistical strength.

Overall, we found no strong evidence of a link between liking (preference) and "wanting" (sign tracking) in our paradigm, which is unsurprising given the general consensus that these two states are comprised of independent systems. (Berridge & Robinson, 2016). Despite initially greater intake levels displayed by the enrichment rats during individual two-bottle tests, increased sign-tracking behavior with extended training did not produce greater ethanol consumption or preference outside the sign tracking procedure. On the flip side, increased sign tracking does not appear to have been driven by a liking of the ethanol solution. From the perspective of sign tracking as a compulsive-like response, it makes sense that the behavior would persist even in the absence of any actual pleasure derived from consuming the solution.

Limitations and Future Directions. Due to lab and time constraints, we chose to limit individual two-bottle choice tests to twenty minutes per rat. We found that the rats did not consume large enough amounts of either substance for strong patterns to form. Based on trends we observed from home cage overnight two-bottle tests, it is possible that group differences may emerge in individual tests with an extended duration. Perhaps increasing two-bottle preference tests to an hour would produce more notable findings while also allowing us to analyze patterns exhibited by individual rats.

We had originally planned to record overnight activity around the site of the ethanol sipper using a color sensor. Due to technical challenges as well as the lab closing due to Covid-19, we were unable to analyze these data for the current study. This component may be revisited in future experiments as a way of determining which rats are drinking during the night, while sign-tracking sessions are not in progress. This could help indicate whether rats that primarily display sign-tracking behavior are drinking more than those that primarily display goal-tracking behavior.

An angle of our experiment was to increase the face value of the sign tracking paradigm by housing and testing rats in a more natural setting using environmental enrichment and by using an object CS that is more consistent with an animal model of ethanol use (i.e., a retractable bottle that elicits species-typical approach behavior and permits the intake of alcohol rather than the more arbitrary retractable lever that elicits only approach behavior) . With our design, we saw significant group differences in sign-tracking behavior, however, we cannot positively determine the extent to which our variables influenced these differences. For instance, we know that the enrichment group displayed stronger sign tracking acquisition, but we are not certain whether this may have been caused by rearing in the enriched housing condition, being tested within the enriched home environment, or a combination of both factors. Environmental enrichment has been found to increase sign-tracking tendencies in past experiments in our lab (Casachahua, 2011), and what we can be certain of is that testing within the home cage did not diminish this effect. From this we can infer that sign tracking is not context-specific and may emerge in settings that are more familiar and natural to the animal, similar to how drug taking and seeking in humans is not always constrained to a particular setting. Lingering questions about the specific influence of context on sign tracking may be addressed in future research by increasing the number of groups to include testing enriched rats both inside and outside the home cage.

Our observations have led us to grow more cynical of the characterization of sign tracking as a compulsive-like behavior. While some researchers have expressed skepticism of animal models that represent substance abuse and addiction as a reflexive and habitual disorder (Field & Kirsbergen, 2019), evidence from our experiment seemed to paint sign tracking in another light. Similar to how humans typically have at least a degree of control over their drug-or reward-seeking behavior, it seems as though rats may have some control too, as indicated by the likelihood of their switching back and forth between goal tracking and sign tracking, as well as within-session habituation. In future experiments, we are interested in allowing a more expansive set of behaviors to exist within sign tracking sessions by removing the partition to provide animals access to the entire tower, or by testing animals in groups or pairs. Studying these alternate behaviors may further clue us in on the “compulsive” nature of sign tracking and allow us a closer examination of individual differences that exist within sign tracking.

We believe that group differences observed in our experiment point to the possibility that traditional sign tracking experiments have come short of eliciting the true natural tendencies of the animals being tested. Incorporating environmental enrichment may have facilitated the emergence of behaviors more natural to the species during testing than those that typically exist within a standard laboratory setting. By altering our method to increase the face validity of the model, we may be simultaneously growing closer to uncovering more natural animal behavior as well as more closely encapsulating the human condition, thereby further narrowing the translatability gap of the sign tracking paradigm.

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Appendix A- Tables and Figures

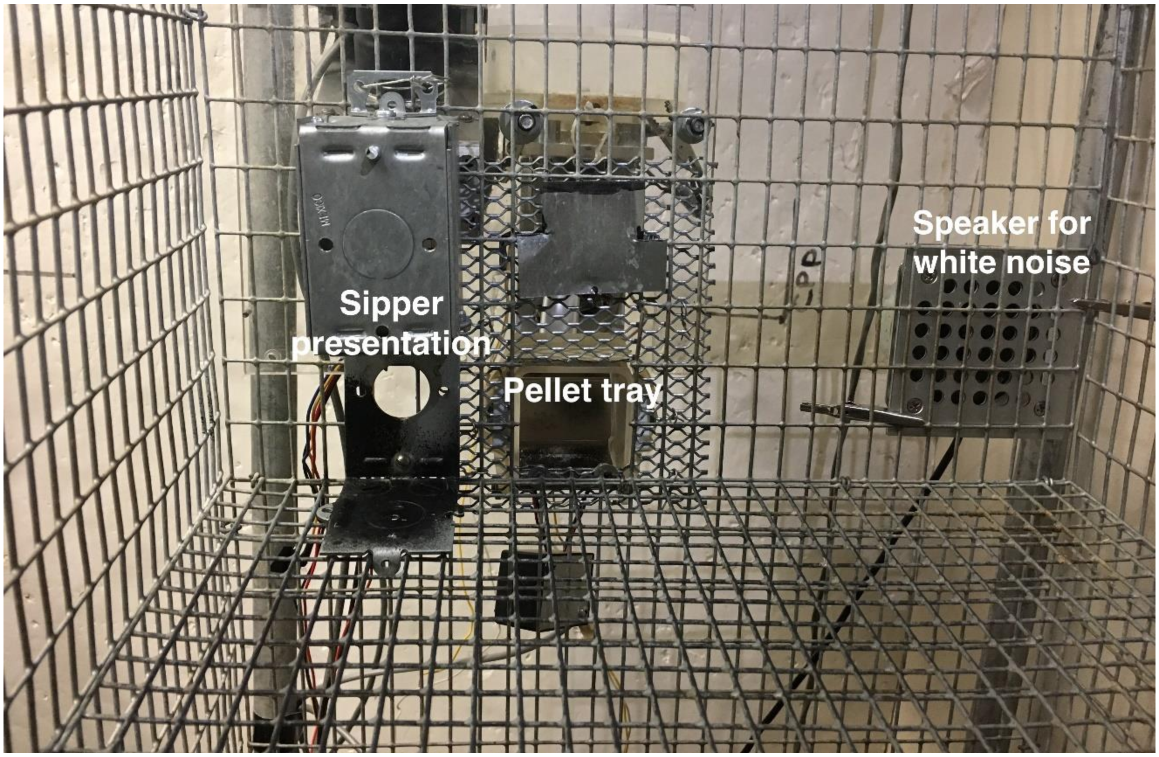

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Table 1. Weekly Schedule

	Wk	Age	M	T	W	R	F	Sa	Su
Arrival/ Adaptation 1	1	21	-	Arrival	Sugar pellets	-	Sugar pellets	-	Sugar pellets
Adaptation Wk 2	2	28	-	Sugar pellets	-	Sugar pellets	2-bottle test(W/W)	-	-
EtOH Prexposure	3	35	2% EtOH	2% EtOH	3% EtOH	-	2-bottle test; 3% EtOH	3% EtOH	3% EtOH
EtOH Prexposure	4	42	4% ETOH	4% ETOH	4% ETOH	-	2-bottle test; 4% EtOH	4% EtOH	4% EtOH
EtOH Prexposure	5	49	5% EtOH / ad	5% EtOH / ad	5% EtOH / ad	ad	2-bottle test; 5% EtOH	-	-
Sign-Tracking Wk 1	6	56	Enr-STd1	Std-STd1	Enr-STd2	Enr-2-bottle test; Std-STd2	Std-2-bottle test; Enr-STd3	Std-STd3; 24h E-Pref	24h E-Pref
Sign-Tracking Wk 2	7	63	Std-STd4	Enr-STd4	Std-2STd5	Std- 2-bottle test; Enr-STd5	Enr-2-bottle test; Std-STd6	Enr-STd6; 24h E-Pref	24h E-Pref
Sign-Tracking Wk 3	8	70	Enr-STd7	Std-STd7	Enr-STd8	Std-SdT8	Enr-STd9	Std-STd9; 24h E-Pref	24h E-Pref
Sign-Tracking Wk 4	9	77	Std-STd10	Enr-STd10	Std-STd11	Enr-STd11	Std-STd12	Enr-STd12; 24h E-Pref	24h E-Pref
Sign-Tracking Wk 5	10	84	Std-2-bottle test; Enr-STd13	Enr-2-bottle test; Std-STd13	Enr-STd14	Enr-2-bottle test; Std-STd14	Std-2-bottle test; Enr-STd15	Std-STd15; 24h E-Pref	24h E-Pref
Sign-Tracking Wk 6	11	91	Std-STd16	Enr-STd16	Std-STd17	Enr-STd17	Std-STd18	Enr-STd18; 24h E-Pref	24h E-Pref

Note: Enr = Enriched (Tower) Group; Std = Standard Housing Group; ST = Sign Tracking procedure; d = day of training
2-bottle test = 20 min tests in separate room, all rats; TBD - to be determined
ad = adaptation to the Sign Traking platform
24h E-Pref = 24 hour Preference Test EtOH available (bottles on center floor for Enr rats)