

**Effects of environmental enrichment on probabilistic reversal learning and motor stereotypy in an animal model of restricted, repetitive behavior**



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## **ABSTRACT**

Restricted, repetitive behavior (RRB) is part of the diagnostic criteria for autism spectrum disorder (ASD) and is characteristic of other neurodevelopmental, neurological, and psychiatric disorders (e.g., Prader-Willi syndrome, Tourette syndrome, and obsessive-compulsive disorder). RRB in ASD includes sensory-motor behaviors (e.g., hand-flapping and body rocking) and behavioral inflexibility (e.g., compulsions and rituals). Animal models are useful in studying repetitive behavior and the neural circuitries that mediate them. The C58 inbred mouse strain engages in motor stereotypy in the form of vertical jumping and backward somersaulting and has shown deficits in a positional discrimination reversal learning task. Studies have shown that environmental enrichment (EE) attenuates repetitive motor behavior, but the effects of EE on behavioral inflexibility in this model have yet to be examined. The present study aimed to explore the effects of EE on the behavioral inflexibility of C58 mice compared to standard housed (SH) mice through the implementation of a probabilistic reversal learning task. It was found that EE significantly reduced repetitive motor behavior, which is consistent with previous findings, and modestly attenuated behavioral inflexibility. These results suggest that EE may be beneficial in reducing behavioral inflexibility in ASD, but further research is warranted. Further studies should examine the neurobiological mechanisms underlying repetitive behavior, as well as sex differences.

**Keywords:** repetitive behavior, autism spectrum disorder, reversal learning, C58, environmental enrichment

## 1. INTRODUCTION

### **Restricted, Repetitive Behavior in Clinical Disorders**

Restricted, repetitive behavior (RRB) refers to sensory-motor actions as well as behaviors reflecting resistance to change that are performed repeatedly in the same way and do not serve an obvious purpose. Examples of sensory-motor behaviors include stereotyped motor movements (e.g., hand flapping and body rocking), behaviors inflicting self-injury, and repeatedly manipulating objects. Repetitive behaviors reflecting resistance to change include compulsions and rituals (Whitehouse & Lewis, 2015).

RRB is commonly observed in various neurodevelopmental disorders (e.g., Prader-Willi syndrome, non-syndromic intellectual and developmental disability) psychiatric disorders (e.g., schizophrenia and obsessive-compulsive disorder) and neurological disorders (e.g., Parkinson's disease and Tourette syndrome) (Bodfish, Symons, Parker & Lewis, 2000; Moss, Oliver, Arron, Burbidge & Berg, 2009). The disorder most relevant to this study is autism spectrum disorder (ASD), for which RRB is one of the diagnostic criteria (Lewis & Bodfish, 1998).

RRB occurs in typically developing young children in the form of repetitive motor movements, commonly before the age of 2 years old (Kim & Lord, 2010) and in the form of rituals, compulsions, and insistence on sameness, most commonly at ages 2 - 4 (Evans et al., 1997). Children often engage in bedtime routines (e.g., reading the same story repeatedly), and have strong preferences for certain foods and clothing items (Evans et al., 1997). As children mature, they steer away from these RRBs and exert more energy on behaviors that serve purposes and help them adapt to their environment. However, rigid and inflexible behaviors persist in people with ASD and often create barriers in their daily lives (Langen, Kas, Staal, Engeland & Durston, 2010). For example, in a room full of diverse toys, a child with ASD will

likely find one toy that he/she finds interesting and will play with that the entire time. This is known as restricted object use and is common among children with ASD (Bruckner & Yoder, 2007). The child will likely get upset if prompted to move away from that toy and play with something else.

Repetitive behavior can hinder learning and social adaptation (Leekam, Prior & Uljarevic, 2011) and serve as a major source of stress for family members (Lord & Jones, 2012). Thus, it is imperative to gain a deeper understanding of these repetitive behaviors in order to improve the quality of life of people with ASD.

### **Animals Models of RRB**

Animal models are particularly useful in the study of repetitive behavior because external variables can be controlled and brains can be examined. This provides opportunities to explore the neurobiological basis of RRB, including relevant neural circuitries. There are many animal models that display sensory-motor repetitive behavior in the form of excessive grooming, pacing, and other rhythmic body movements (Bechard & Lewis, 2012). The C58 mouse strain is one of these models.

C58s are an inbred mouse strain that display repetitive vertical jumping and backward somersaulting (Ryan, Young, Crawley, Bodfish & Moy, 2010), behaviors that are analogous to the sensory-motor repetitive behaviors seen in people with ASD. These behaviors in C58 mice occur spontaneously in the early stages of development and continue over time (Muehlmann et al., 2012). This mouse strain also shows social deficits (Ryan, Young, Crawley, Bodfish & Moy, 2010), which increases its validity as a model relevant to ASD. Behavioral inflexibility or resistance to change is more difficult to operationalize in animals than repetitive sensory-motor actions. However, assessing perseveration in reversal learning tasks has proven to be a useful

way of measuring resistance to change in animal models (Bechard & Lewis, 2012). C58 mice have been shown to be impaired in reversal learning tasks similar to the one used in the present study (Whitehouse, Curry-Pochy, Shafer, Rudy & Lewis 2017). In this study, C58 mice were unable to reverse their pattern of responding when reinforcement for responding on one side of the cage was shifted to the other side.

A similar study explored behavioral inflexibility in the BTBR mouse strain, a different mouse model of ASD that exhibits repetitive motor behavior in the form of excessive grooming (Amodeo, Jones, Sweeney & Ragozzino, 2012). It was found that the BTBR mice showed deficits in reversal learning of a spatial discrimination task, as well as increased sensory-motor repetitive behavior (excessive grooming and marble burying) when compared to a control mouse strain (Amodeo, Jones, Sweeney & Ragozzino, 2012).

### **Environmental Enrichment**

Repetitive behavior in animals can be induced through genetic mutations, pharmacological agents, and environmental restriction (Bechard & Lewis, 2012). Examples of repetitive behaviors resulting from restricted environments include pacing in zoo animals, bar-biting in farm animals, and route-tracing in laboratory animals (Mason & Rushen, 2008). The repetitive motor behavior of the C58 mice appears to be the result of an interaction between their genotype and the restricted environment in which they are reared. Confined settings (Mason and Rushen, 2008) and early social deprivation (H. Harlow & M. Harlow, 1962) are associated with repetitive behaviors across various species, including humans. In the early 1990s, the majority of adopted Romanian orphans displayed repetitive behavior, mostly in the form of body rocking (Fisher, Ames, Chisholm & Savoie, 1997). This stereotypic behavior has been attributed to

inadequate interpersonal contact and lack of sensory stimulation in the Romanian orphanages (Wilson, 2003).

Environmental enrichment (EE) refers to the addition of toys, levels and materials to laboratory cages, which increases sensory stimulation, and cognitive and physical activity (Hannan, 2014). EE has significant positive implications on the brain, such as increasing the growth and development of neurons and synapses (van Praag, Kempermann & Gage, 2000). EE has been shown to reduce sensory-motor repetitive behavior across several species (Mason, Clubb, Latham & Vickery, 2007), including C58 mice (Muehlmann et al., 2012). , The effects of EE on resistance to change have not yet been adequately addressed in the C58 model. However, previous work from our lab showed that EE improved reversal learning in deer mice (Tanimura, Yang & Lewis, 2008). In addition, EE was shown to reduce perseverative responding in mink (Campbell, Dallaire & Mason, 2013) and has been shown to enhance reversal learning in C57BL/6 mice (Zeleznikow-Johnston, Burrows, Renoir, & Hannan, 2017).

### **Purpose of Present Study**

A recent study provided preliminary evidence that EE may attenuate behavioral inflexibility in C58 mice, however, only four female mice were studied so further research is clearly warranted (Whitehouse, Curry-Pochy, Shafer, Rudy & Lewis, 2017). The present study aimed to explore behavioral inflexibility in the C58 mouse strain by implementing reversal learning of a positional discrimination task. Specifically, standard housed mice were compared to environmentally enriched mice to examine the effect of housing on repetitive behavior. It was hypothesized that C58 mice housed in EE would exhibit less behavioral inflexibility (better reversal learning) than C58 mice housed in the SH condition.

Further evidence of behavioral inflexibility would broaden the extent to which the C58 mouse strain models the larger range of repetitive behavior in clinical disorders such as autism. This will improve the translational value of the C58 model. Particularly, studying the effects of EE on behavioral inflexibility may provide significant implications for its use in decreasing behavioral inflexibility in people with ASD.

## **2. MATERIALS AND METHODS**

### **2.1 Animals**

C58 mice (N=40) of both sexes were bred and housed at the University of Florida. Humidity and temperature were controlled in the colony with 50-70% humidity and temperature within the range of 70-75°F. The colony room was maintained on a 12:12 light:dark cycle with lights off at 8:00 pm. All C58 mice were used for assessment of repetitive motor behaviors and reversal learning tasks.

After weaning at postnatal day 21, C58 mice were randomly assigned to either standard housing or environmentally enriched housing. Both types of housing were kept in the same room.

In the standard housing condition, 29 C58 mice (13 males, 16 females) were housed with one to three other C58 mice of the same sex in standard laboratory cages (29 x 18 x 13 cm). Water was available *ad lib* and two Nestlet squares were provided for nest construction.

In the environmental enrichment condition, 11 C58 mice (8 males, 3 females) were housed with two to three other C58 mice of the same sex in a large dog kennel (121.9 x 81.3 x 88.9 cm) modified with two extra levels and connected by ramps constructed from galvanized wire (Muehlmann et al., 2012). Throughout the 5-week enrichment period, a running wheel, a

large opaque shelter, and Habitrail tubes were kept in the kennel. Various objects, such as plastic toys and domes, were placed in the kennel, but rotated weekly to maintain novelty. While objects were rotated out each week, approximately 2 oz. of birdseed was scattered into the kennel to promote foraging. Water was available *ad lib* and four Nestlet squares were provided for nest construction.

After 5 weeks in environmental enrichment and following assessment of motor stereotypy, mice were placed into a modified enriched environment to facilitate handling during testing. The modified enrichment cage was the same size as the standard housing cage (29 x 18 x 13 cm) and included a running wheel, a shelter, and 2 toys that were rotated each week. Water was available *ad lib* and two Nestlet squares were provided. The modified enrichment cages were kept in the same room as the standard housed animals.

Animal care and use was performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the University of Florida Institutional Animal Care and Use Committee.

## **2.2 Equipment**

Photobeam arrays (Columbus Instruments) were used to quantify the motor stereotypies in C58 mice. Repetitive motor behavior of C58 mice is expressed as vertical hindlimb jumping and backward somersaulting. Mice were individually tested in clear plexiglass chambers (28 x 22 x 25 cm). Vertical jumping and backward somersaulting interrupted the photobeams, which were then recorded as counts with attached time stamps. Rearing and other non-stereotyped vertical activity did not interrupt the photobeam and therefore did not influence quantification. Video recordings of all test sessions were used to identify the topography of repetitive motor behavior and to verify the accuracy of the automated counters.

To assess reversal learning, operant chambers (Med Associates model ENV-307W, 21.6 x 17.8 x 12.7 cm; St. Albans, VT) were used. The chambers were enclosed in sound attenuating cabinets and were equipped with two nose-poke holes, which could be illuminated with cue lights (ENV-314W with a diameter of 1.3 cm and a depth of 1 cm). The food hopper, which released 14 mg Dustless Precision Pellet®: Rodent, Purified (Bio-Serv®), was located between the two nose-poke holes. Programming was written and data was collected using Med-PC IV software (Med Associates, St. Albans, VT). Video recording was used to verify the accuracy of the software and to examine additional behaviors while in the operant chamber. Between each session, each operant chamber was cleaned with 70% ethanol.

### **2.3 Repetitive Motor Behavior**

Before testing, all mice were assessed for their repetitive motor behaviors using the photobeam arrays previously described. Between postnatal day 56 and 63, mice were placed in individual test chambers for one hour prior to testing. After habituation, each mouse was assessed during the 12-hour dark cycle with food and water available *ad lib*.

### **2.4 Experimental Design**

This study was designed to test the hypothesis that C58 mice housed in an enriched environment would exhibit reduced repetitive motor behavior and more evidence of behavioral flexibility than C58 mice housed in a standard environment. To test this hypothesis, we compared two groups of mice: Standard Housing (SH) and Environmental Enrichment (EE). All mice were subjected to a right/left positional discrimination task and, upon meeting criterion for acquisition, underwent reversal learning.

## 2.4a Acquisition

Before operant testing, the average weight of each mouse was calculated and access to food was adjusted to reduce mouse body weights to 85-90% of the average free feeding weight. All operant testing occurred during the light cycle between 9:00 am and 4:00 pm. None of the mice had previous experience with operant testing. Prior to the start of each 20 minute session, the mice underwent a habituation session of 10 minutes in the operant chamber with the house light off. Mice were randomly assigned to be reinforced on the right side or the left side during acquisition. Nose pokes were reinforced with a 14 mg Dustless Precision Pellet®: Rodent, Purified (Bio-Serv®). When a mouse responded on the correct side, a food reward pellet was delivered to the food hopper, the yellow cue lights that illuminated the two nose-poke holes turned off to indicate that they were inactive, and a click tone sounded to signify the delivery of the reinforcer. Once the food was retrieved from the hopper, the nose-poke holes became active and illuminated again. Animals were free to explore the chamber and interact with the nose-poke holes on their own, so no formal shaping occurred. Before the first session, a food pellet from the home cage was rubbed around the nose poke holes to increase the probability of interaction.

Both groups of mice (SH and EE) began with fixed ratio 1 (FR1) schedule of 100% reinforcement on one side and no reinforcement on the opposite side. The response criterion was set at 20 total responses with 50% of responses on the reinforced side. If an animal did not meet this criterion within six sessions, it was dropped from the study. After FR1 acquisition, both groups were switched to a probabilistic reinforcement schedule where 80% of responses were now reinforced on the side that was previously reinforced 100% of the time, and 20% of responses were now reinforced on the side that was previously unreinforced. The acquisition

criterion set was 75% of all nose-pokes reinforced for one session. If an animal did not meet criterion within six sessions, it was dropped from the study.

### **2.4b Reversal Learning**

After the acquisition criterion was met, both groups began the reversal learning condition. During reversal learning, the location of reinforcement was reversed from the previously reinforced side during acquisition (80% reinforcement side was now 20% reinforced and vice versa). The mice completed 5 sessions of reversal learning. Based on the findings of Whitehouse, Curry-Pochy, Shafer, Rudy & Lewis (2017), it was expected that significant differences would be apparent by day 5. This also makes the protocol manageable for routine use.

### **2.5 Statistical Analyses**

To analyze the differences between the Standard Housing and Environmental Enrichment groups, independent sample t-tests and repeated measures analysis of variance (RM-ANOVA) were conducted. Independent sample t-tests were performed to compare the effects of housing on the frequency of motor stereotypy and the number of sessions and responses emitted to reach acquisition criterion.

A Repeated Measures Analysis of Variance (RM-ANOVA) was conducted to examine the housing differences in the responses on the high reinforcement density side as a proportion of total responses during reversal learning and the percent of responses reinforced during reversal learning.

The relationship between repetitive motor behavior and measures of the positional discrimination task was analyzed with Pearson correlation or Spearman rank where applicable. The measures of positional discrimination consisted of the number of responses for the acquisition and reversal learning sessions, the number of responses to criterion for acquisition

and reversal, and the number and proportion of responses on the high reinforcement density side as a proportion of total responses in reversal learning. For all statistical analysis, differences were considered significant when  $p < 0.05$ .

### 3. RESULTS

#### 3.1 Repetitive Motor Behavior

Standard housed (SH) mice exhibited significantly more repetitive motor behaviors than environmentally enriched (EE) mice,  $t(24.44) = 6.42, p < 0.001$  (Figure 3-1).

#### 3.2 Acquisition

There were no significant differences between SH and EE groups in the number of sessions needed to meet acquisition criterion,  $t(33) = 1.08, p = 0.29$ , or in the number of responses needed to meet acquisition criterion,  $t(33) = 0.06, p = 0.95$  (Figure 3-2).

In the EE group ( $n = 11$ ; 8 males, 3 females), one male was dropped after not meeting acquisition criterion. In the SH group ( $n = 29$ ; 13 males, 16 females), two males were dropped after not meeting acquisition criterion and two females were dropped due to operant box malfunctioning. The greater number of SH mice was due to their involvement in a larger study.

#### 3.3 Reversal Learning

A 2-factor GLM RM-ANOVA was used to compare housing condition differences with the proportion of responses on the high reinforcement density side across 5 reversal learning (RL) sessions (Figure 3-3 A). There was a main effect of reversal learning session,  $F(2.53, 80.9) = 24.3, p < 0.001$ , and a reversal learning session x housing condition interaction,  $F(2.53, 80.9) = 2.85, p = 0.05$ . There was no main effect of housing condition,  $F(1, 32) = 2.16, p = 0.15$ . There were no significant differences between housing conditions across the first 4 sessions of RL, but

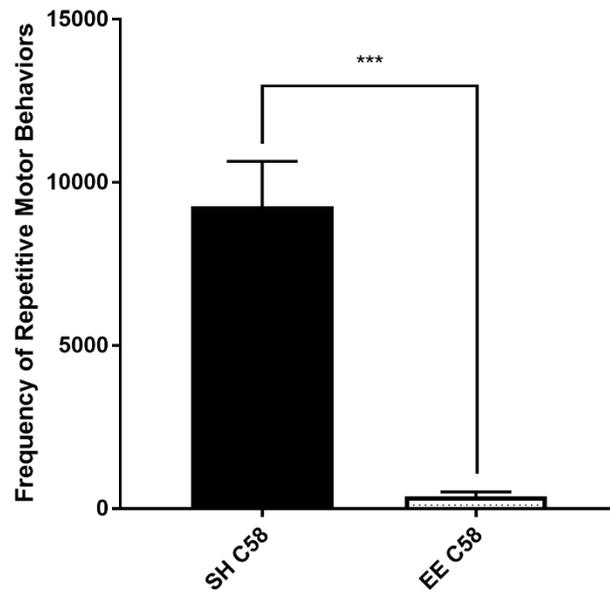
EE mice emitted significantly more responses on the high reinforcement density side by session 5,  $t(33) = 2.03$ ,  $p=0.05$ . Group differences on day 5 of reversal learning can be seen in Figure 3-4.

A 2-factor GLM RM-ANOVA was also used to compare housing condition differences with the proportion of responses reinforced across the 5 reversal learning sessions (Figure 3-3 B). There was a main effect of reversal learning session,  $F(2.43, 73.0) = 25.6$ ,  $p < 0.001$ , but no main effect of housing condition,  $F(1, 30) = 2.14$ ,  $p = 0.15$ , or reversal learning session x housing condition interaction,  $F(2.43, 73.0) = 2.61$ ,  $p = 0.07$ . Across reversal learning sessions, mice in both housing conditions showed increases in the proportion of responses reinforced.

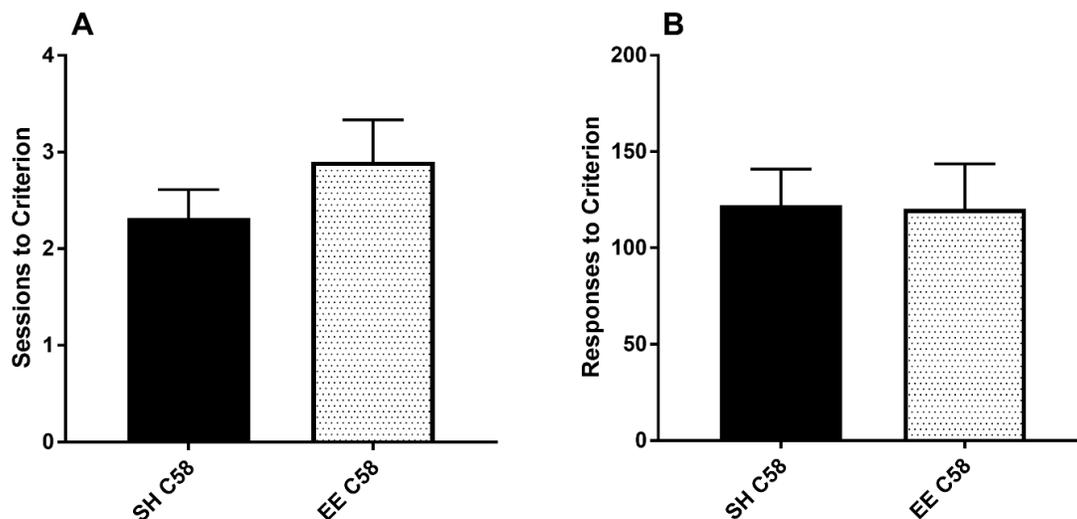
The lose-stay measure quantifies behavioral inflexibility by recording continued responses on the same side despite lack of reinforcement. There was a significant difference in the proportion of lose-stay responses across reversal learning sessions 1 and 5 (Figure 3-5). A 2-factor GLM RM-ANOVA showed that there was a significant main effect of reversal learning session,  $F(1, 33) = 59.8$ ,  $p < 0.001$ , with decreases in the proportion of lose-stay responses in both housing conditions by session 5. There was no main effect of housing condition,  $F(1, 33) = 0.80$ ,  $p = 0.38$ , but there was a significant reversal learning session x housing condition interaction,  $F(1, 33) = 9.63$ ,  $p = 0.004$ . EE mice reduced their lose-stay responses more than SH mice.

Spearman's rho was used to analyze correlations between frequency of repetitive motor behavior and operant measures. In both housing conditions, there was a correlation between frequency of repetitive motor behavior and percent of responses reinforced on day 5 of reversal learning,  $r(33) = -0.38$ ,  $p = 0.03$ . There was also a correlation between frequency of repetitive motor behavior and percent of responses on the high reinforcement density side on day 5 of reversal learning,  $r(33) = -0.35$ ,  $p = 0.04$  (Figure 3-6). Although this correlation was significant,

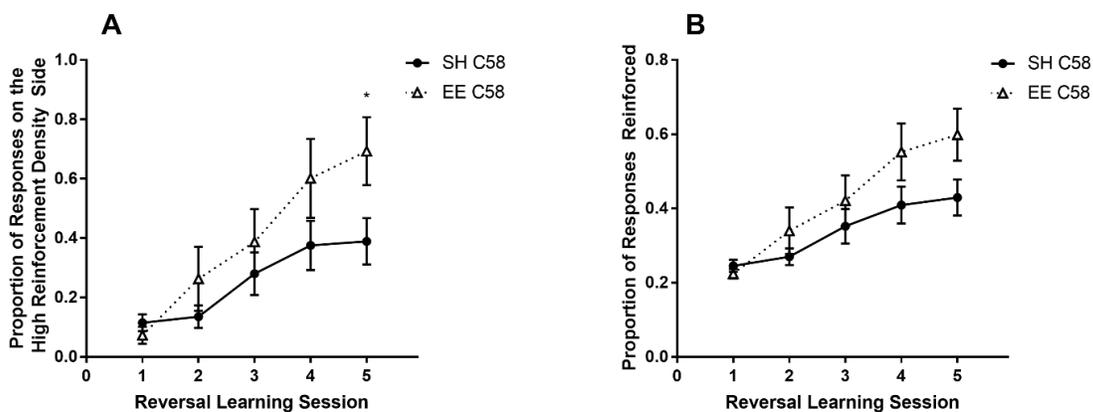
the scatterplot does not show evidence of a clear trend and the percent of variance shared (<15%) was low. Therefore, this statistic should be addressed with caution.



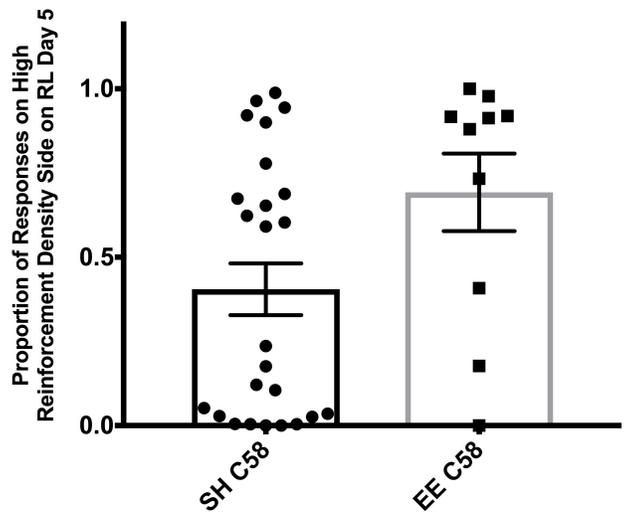
**Figure 3-1** Housing condition differences in repetitive motor behavior. SH mice exhibited significantly more repetitive motor behaviors than EE mice.



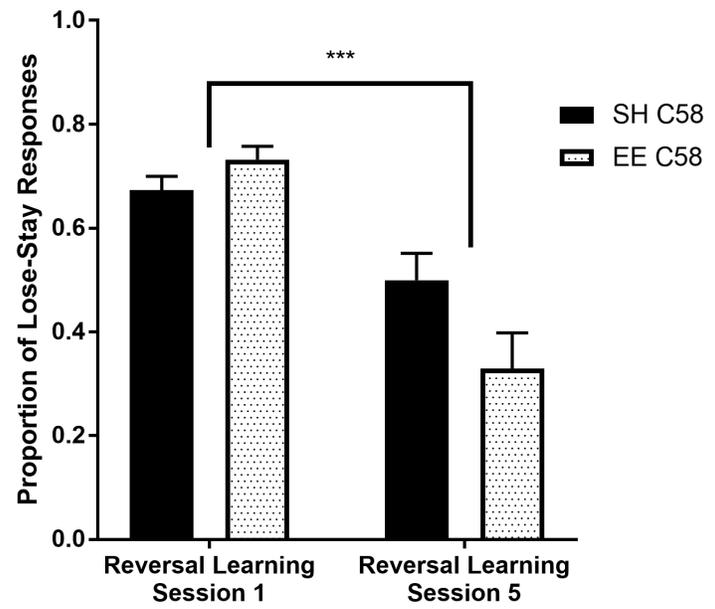
**Figure 3-2** Housing condition differences in acquisition. There were no significant differences between housing conditions in acquisition. The SH and EE groups completed a similar number of sessions and emitted a similar number of responses to meet acquisition criterion.



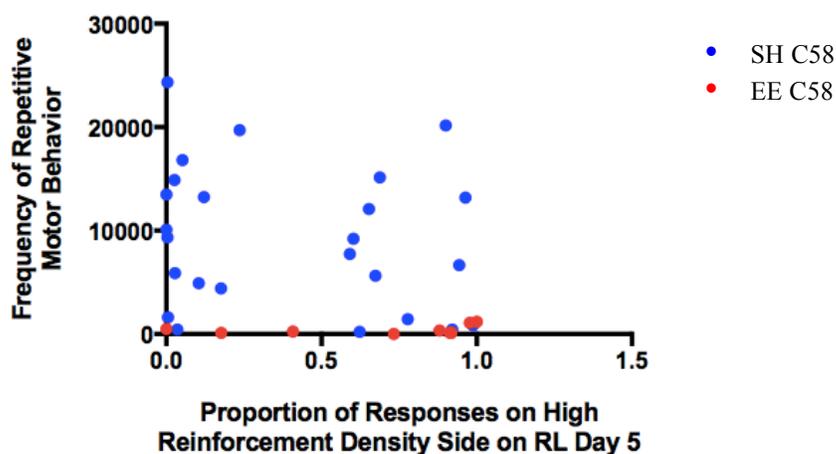
**Figure 3-3** Housing condition differences in reversal learning. (A) EE mice emitted more responses on the high reinforcement density side than SH mice across the 5 sessions of reversal learning. (B) EE and SH mice received a similar proportion of reinforcement in reversal learning.



**Figure 3-4** Group differences in proportion of responses on the high reinforcement density side on day 5 of reversal learning.



**Figure 3-5** Housing condition differences of lose-stay responses in reversal learning. SH and EE mice reduced the number of lose-stay responses across the 5 reversal learning sessions, but EE mice reduced these responses more. There was no main effect of housing condition, but there was a significant interaction of housing x session.



**Figure 3-6** Correlation between frequency of repetitive motor behavior and percent of responses on the high reinforcement density side on day 5 of reversal learning. The lack of a clear trend shows that this correlation is spurious.

#### 4. DISCUSSION

The present study was designed to examine the effects of environmental enrichment on behavioral inflexibility in C58 mice through implementation of a positional discrimination task with a reversal learning phase. It was hypothesized that environmental enrichment would reduce behavioral inflexibility (e.g., improve reversal learning) as seen in the pilot study by Whitehouse, Curry-Pochy, Shafer, Rudy & Lewis (2017).

As expected, environmental enrichment attenuated repetitive motor behavior (vertical jumping and backward somersaulting) in C58 mice. This is consistent with the literature, specifically with the findings of Muehlmann et al. (2012). There were no significant differences observed during acquisition, which rules out deficits in initial learning of the task as an explanation for behavioral differences during the reversal learning phase. The lack of differences in acquisition was a methodological strength, but is inconsistent with the literature, which

suggests that environmental enrichment generally enhances learning (Cortese, Olin, O’Riordan, Hullinger & Burger, 2018; Hullinger, O’Riordan & Burger, 2015). A recent study found that environmentally enriched mice learned an Olfactory Temporal Order Discrimination task significantly faster than standard housed mice (Rountree-Harrison, Burton, Leamey & Sawatari, 2018).

Environmentally enriched mice performed significantly, albeit modestly, better in the reversal learning task than standard housed mice, as predicted based on the findings of Whitehouse, Curry-Pochy, Shafer, Rudy & Lewis (2017). While environmentally enriched mice responded more on the high reinforcement density side and were reinforced more during reversal learning, the difference compared to standard housed mice was modest. Environmentally enriched mice also showed a significant reduction in lose-stay responses compared to standard housed mice.

Limitations of this study include an uneven distribution of sex, a smaller environmental enrichment group, and the brevity of the number of sessions. The environmental enrichment group had 11 mice, with only 3 females, while the standard housed group had 29 mice and a more even distribution of males and females. Replication studies should have larger sample sizes with equal numbers in each group and an even distribution of males and females. Increasing the number of days spent learning the task and in reversal learning would also yield more valid data.

The effects of environmental enrichment on behavioral inflexibility in C58 mice were unexplored in the literature prior to the preliminary findings of Whitehouse, Curry-Pochy, Shafer, Rudy & Lewis (2017), however, this study only involved four female mice. The present study was the first to replicate that study. The novelty of this research question and the

differences found between housing conditions warrants the need for continued research in this area.

At this point, environmental enrichment appears to significantly attenuate sensory-motor repetitive behavior and to modestly attenuate behavioral inflexibility in C58 mice. Perhaps this difference can be attributed to differentiations in the neural circuitries mediating these pathways. Future research should examine the neurobiology associated with each cluster of repetitive behavior in C58 mice. Future studies should also examine sex differences in C58 mice because there are significant sex differences in people with ASD. Boys are 4.5 times more likely to be diagnosed with ASD than girls (Christensen et al., 2016) and show more repetitive and stereotypic behaviors (Hartley & Sikora, 2009; Lord, Schopler & Revicki, 1982). This would increase the translational value of this mouse model to humans.

Restricted, repetitive behavior can negatively influence the lives of people with ASD. A recent study found that 71% of a sample of school-aged children had difficulty adapting to their environment because they were locked into performing repetitive behaviors (Cashin & Yorke, 2018). Understanding the neurobiology that drives repetitive behavior and the implications of environmental enrichment are vital in the development of cognitive-behavioral therapies and pharmacological agents to improve the adaptive abilities and thus quality of life of people with ASD. Two studies have shown that environmental enrichment significantly improved cognitive functioning and symptom severity in children with ASD (Woo & Leon, 2013; Woo, Donnelly, Steinberg-Epstein & Leon, 2015). Children displayed increased IQ scores, improved abilities in receiving language, and reductions in atypical responses to sensory stimuli (Woo, Donnelly, Steinberg-Epstein & Leon, 2015). These studies illustrate the potential that environmental

enrichment has to improve the lives of people with ASD. Continued research in this area could expand these therapies and increase their efficacy.

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