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The Attention Set-Shifting Test is Sensitive for Revealing Sex-based Impairments in Executive Functions Following Developmental Lead Exposure in Rats

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Abstract

The literature on lead (Pb) exposure has focused in large part on hippocampal-based learning and memory deficits, although frontoexecutive dysfunctions are known to exist in Pb-exposed humans. This study examined the effects of perinatal (PERI) and early postnatal (EPN) developmental low-level Pb-exposures in rats on frontoexecutive functions, using the Attention Set-Shift Test (ASST). Control males and females performed the ASST similarly. Male EPN rats had difficulty with simple discrimination (SD) of odors and failed to complete the compound discrimination (CD) stage of the ASST. All other Pb-exposed rats completed the training and testing. Male PERI rats performed worse on the SD, intradimensional (ID), and intradimensional-reversal (ID-Rev) ASST stages when compared to male Control rats. Female EPN rats performed similar to Controls on the ID-Rev, whereas PERI rats performed better the trials-to-criterion on the ID-Rev than EPN and Control rats. Pb-exposed female rats had significant difficulty performing the ED/ED-Rev stages, with the number of trials-to-criterion double that required by Pb-exposed and Control male rats and Control female rats. Together, the ASST results showed that developmental Pb-exposure induces frontoexecutive dysfunction that persists into adulthood, with different sex-based vulnerabilities dependent upon the time-period of neurotoxicant exposure.

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5.0 Author's Contributions

Conceived and designed the experiments: LSN, DWA, and JSS. Performed the BLL collection and analyses, as well as, the behavioral experiments: LSN. Analyzed the data: LSN and SM. Wrote the manuscript: LSN and JSS.

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6.0 Conflicts of Interest

LSN discloses a public domain trademark used under common law as the Neuwirth-ASST™ Apparatus. Otherwise, the authors declare no other conflicts of interest.

Keywords

Attention Set-Shifting Test; Developmental Lead Exposure; Frontoexecutive Functions; Dysexecutive Functions; Cognitive flexibility; dimensional shifts

1.0 Introduction

The attention set-shift test (ASST) for rodents has become an increasingly valuable tool for assessing frontoexecutive functions to model human frontal lobe disorders, dementias, and others disease states (for review see Tait, Bowman, Neuwirth & Brown, 2018; Bissonette, Powell & Roesch, 2013). The ASST has proved to be a sensitive and reliable neurobehavioral test capable of distinguishing between differences in cognitive (in)flexibility, attentional shifting or lack thereof, reversal learning abilities and impairments, as well as the presence or absence of perseverative behaviors in lesion models of frontoexecutive dysfunction (Gallagher, Stocker & Koh, 2011; Bissonette et al., 2008; Fox, Barense & Baxter, 2003; Barense, Fox & Baxter, 2002; Birrell & Brown, 2000). The ASST has also been used to assess frontoexecutive functions in animal models of attention-deficit/hyperactivity disorder (AD/HD) (Aronn & Poldrack, 2005), stress (Amsten, 2009), emotional fear (Gilmarating, Balderston & Helmstetter, 2014), and schizophrenia. Further, Dalley et al. (2004) revealed how sub-regions of the prefrontal cortex and striatum may differentially and cooperatively contribute to working memory and learning within the ASST and its generalization to executive function across mammals (for review see Bissonette, Powell & Roesch, 2013; Bizon, Foster, Alexander & Glisky, 2012; Chudasama & Robbins, 2006).

Longitudinal studies have found that children with blood Pb levels (BLLs) above 5 µg/dL at preschool age and followed into adulthood (*i.e.*, ~19–24 years of age), have sex-based differences (*i.e.*, males more affected than females) in left anterior cingulate, right superior frontal, and medial frontal gyri volumetric loss (Cecil et al., 2008). Environmental Pb-exposure in children during early development has also been shown to result in persistent impairments in several frontal cortical-mediated cognitive domains (Lidsky & Schneider, 2006; Lidsky & Schneider, 2003). Despite findings of executive dysfunction in Pb-exposed children (Roy et al., 2009; Canfield, et al., 2005; Canfield, Gendle & Cory-Slechta, 2004; Canfield et al., 2003), frontoexecutive deficits in animal models of developmental Pb-exposure have not been examined with tests such as the ASST.

Developmental Pb-exposure has been widely documented to produce hippocampal-dependent learning and memory disruptions in animals (see for review Marchetti, 2003; Bresslet et al., 1999; Silbergeld, 1992), with specific outcomes related to postnatal (Alfano & Petit, 1981), perinatal (Baranowska-Bosiacka et al., 2013) and neonatal (Alfano & Petit, 1982) Pb-exposures. Developmental Pb-exposures result in hippocampal disruptions in synaptic plasticity (Lasley & Gilbert, 2000), astrogliosis (Selvin-Testa et al., 1994) and apoptosis (Sharifi et al., 2002). Although the prefrontal cortex is amongst the brain regions most vulnerable to Pb-induced damage (Finklestein, Markowitz & Rosen, 1998) and one of the last brain regions to fully mature; comparatively, little experimental research has been

performed regarding the impact of developmental Pb-exposure on attention and frontoexecutive functions. Additionally, Pb-induced dysfunction of the prefrontal cortex could contribute to hippocampal-associated encoding and retrieval deficits. It was hypothesized that developmental Pb-exposure causes disruptions in prefrontal cortical executive functions that are the foundation for learning and memory cognitive processes that can be examined in the rat model using the ASST. Thus, the present study sought to investigate the effects of different types of developmental time-periods of Pb-exposure, their related influences on frontoexecutive functions long after the rats were removed from the Pb-exposure source (*i.e.*, simulating the clinical study design described by Cecil et al., 2008), and further examined sex-based differences in the rat's cognitive abilities in attentional learning, forming attention-sets, and cognitive flexibility in performance of the ASST.

2.0 Method

2.1 Subjects

The animal procedures complied with National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and the study was approved by the Institutional Animal Care and Use Committee (IACUC) at Thomas Jefferson University. Rats were housed four to a cage (47.6 X 25.9 cm) and were exposed to a 12:12 h light: dark cycle for the duration of the study. Rats were randomly assigned treatment conditions as described previously (Varma et al., 2017; Verma & Schneider, 2017). Briefly, Long Evans dams (Harlan Laboratories) in the perinatal (PERI) group were fed chow (RMH 1000) with or without Pb-acetate (0ppm or 150ppm) (Dyets, Inc., Bethlehem, PA) for two-weeks prior to breeding and remained on the same diet through weaning (postnatal day [PND] 22) in order to assess Pb-exposure influences on the developing rat brain (Semple et al., 2013). Another set of rats were exposed to the same Pb-diet, however the time-period of exposure began on PND 0 and continued to PND 22 (early postnatal exposure group [EPN]). A total of $N = 36$ rats (*i.e.*, $n = 18$ males and $n = 18$ females) were randomly assigned following breeding to the subsequent experimental treatment conditions for each gender: Control $n = 6$, EPN $n = 6$, PERI $n = 6$, consistent with the numbers of animals required for the ASST (for review see Tait, Bowman, Neuwirth & Brown, 2018; Birrell & Brown, 2000). At PND 22, all rats were removed from Pb-diets, and provided regular RMH 1000 chow until the study was completed. To control for litter effects, all litters were culled to an equal number of pups to standardize litter size at eight pups per litter and equal numbers of males and females were maintained wherever possible. One male and female were randomly sampled from each litter and then combined with rats from other litters to form the final experimental cohorts described herein.

2.2 Blood Lead Levels (BLLs)

One male and female pup were randomly sampled, from six different dams, from each treatment group, to determine BLLs. Transcardial blood samples were drawn from the pups and stored in EDTA-containing collection tubes (Sardstedt, Germany). Additionally, blood samples were obtained from the dams by retro-orbital bleeding (ROB) under isoflurane anesthesia before Pb-exposure and at parturition following Pb-exposure. Blood samples were analyzed using an ESA LeadCare II Blood Lead Analyzer system (V. 1.09) with a Lead

Care II Blood Lead Test Kit (Magellan Diagnostics, MA). Briefly, 50 μ L of whole blood was mixed with 250 μ L of diluted hydrochloric acid solution (0.34 M) and was applied to the sensor strip of the Lead Care II Blood Lead Analyzer system, which recorded BLL values in μ g/dL with a lower sensitivity limit of 3.33 μ g/dL and a higher sensitivity limit of 65.00 μ g/dL.

2.3 Experimental Design

2.3.1 Food Restriction & Animal Weights—In order to have animals work for a food reward in the ASST, at PND 55, rats assigned to the ASST were individually housed and placed on a food restricted diet (*i.e.*, 15–20 g for males and 12–15 g for females of rat chow per day) with water provided *ad libitum* consistent with the NIH (2017) Guidelines for Diet Control in Behavioral Studies (<http://oacu.od.nih.gov/ARAC/dietctrol.pdf>). The rats were maintained at 85% of their *ad libitum* body weight to provide sufficient motivation to search for a food reward in order to perform the ASST. If rats lost more than 7–9% of normal body weight within one-week, their diets were adjusted accordingly to prevent excessive weight loss. The food restrictions were initiated two-weeks prior to training to establish a metabolic steady state and to prevent any additional experimental confounds. The weights for each rat were taken as a baseline value prior to being placed on food restriction and was continually monitored, with animals weighed every Monday, Wednesday, and Friday until testing was completed.

2.3.2 Handling & Habituation—Beginning 14 days prior to training, all rats were handled for a minimum of 10-minutes per day. The same researcher handled the rats throughout the study to minimize any stress that could result from handling by different individuals.

2.3.3 Training Procedures—All procedures were performed according to those described by Birrell & Brown (2000). Briefly, rats were taught to dig in bowls by using a gradual shaping procedure that systematically increased the amount of digging medium (*i.e.*, shredded paper) to bury the reinforcer (*i.e.*, half a Froot Loop, Kellogs™). A correct training response was operationally defined as retrieving the Froot Loop from under the digging medium within a 10-minute trial. The criteria for moving to the next training stage was to complete 6-consecutive correct responses. If a rat did not retrieve the Froot Loop within the 10-minute allotted time-period, it was defined as an error. Trials were repeated until each rat was able to complete the training stage and advance onward to the ASST testing stages. Each bowl was lightly coated with the dust from crushed Froot Loops from a range of colors to eliminate any odor bias in finding the food reward. Moreover, each trial was presented with a different color Froot Loop so the rat would not fixate on a particular colored Froot Loop; thereby, preventing any additional experimental confounds. Rats moved through the training phase by digging through the medium and obtaining the Froot Loop on 6-consecutive trials in each of the 5-stages with bowls filled with increased amounts of digging medium (*i.e.*, with the bowls filled with 0%, 25%, 50%, 75%, and 100% of the digging medium, respectively). This initial training was completed in a single test session within one day.

2.3.4 Testing Procedures—As in the training stage, the criterion to advance through the ASST was 6-consecutive correct responses. If a rat were to make an incorrect response (*i.e.*, an error), the consecutive count would be reset, while the number of trials-to-criterion [TTC] and the number of errors-to-criterion [ETC] were recorded. If a rat had 6-consecutive incorrect responses, they failed the ASST test stage for that day. The rats were re-tested for up to 3-consecutive days. If the rats still did not complete the ASST stage, this constituted an exclusory criterion from the study. The pairs of testing stimuli used are shown in Table 1. All testing procedures were conducted as described by Birrell & Brown (2000) with the following modifications. Due to the Pb-exposure, the PERI and EPN rats exhibited elevated levels of neophobia and stress compared to Control rats. Thus, in order to decrease the PERI and EPN rats neophobia and stress and increase their ASST performance in a well-controlled manner, the 7-stage ASST was split into the following 3-day 3-stage test procedure: Test Day # 1 included the simple discrimination (SD), complex discrimination (CD), and complex discrimination reversal (CD-Rev); Test Day # 2 included a reacquisition probe of the complex discrimination reversal (CD-ReAcq), the intradimensional shift (ID), and the intradimensional shift reversal (ID-Rev); and Test Day # 3 included a reacquisition probe of the intradimensional reversal (ID-ReAcq), the extradimensional shift (ED), and the extradimensional shift reversal (ED-Rev). The CD-ReAcq and ID-ReAcq conditions were used to ensure the stability of responding across days prior to instituting the next test stage condition in order to maintain an appropriate behavioral momentum in all rats.

2.3.5 Anymaze Video Tracking—The ASST training and testing phases for each rat were video recorded and analyzed using Anymaze™ software (Stoelting, Inc.; Wood Dale, IL). Within the Anymaze video tracking software, a novel experimental program was written (Neuwirth-ASST™) to track TTC, ETC, latency to respond, and the number of perseverative responses exhibited within the ASST. In this study, perseverative responses were defined as the number of errors that occurred within and between the four discovery trials within the CD-Rev, ID-Rev, and ED-Rev, respectively. The discovery trials consisted of the rats being permitted to search the other bowl to learn the proper odor or digging medium association with the Froot Loop if they made an incorrect response within the 10-minute training or testing trial (for review see Birrell & Brown, 2000). Notably, since the TTC data showed the same pattern as the ETC data, for simplicity the TTC data are only reported herein.

2.4 Statistical Analyses

The ASST was separated into three experiments. First, *Experiment 1* evaluated whether there were any sex differences between Control male and Control female rats in performing the ASST. Next, *Experiments 2* and *3* evaluated whether the effects of developmental Pb-exposure differentially influenced males and female ASST performance, respectively. Data were analyzed using SPSS (V.24) and employed a two-way repeated measures factorial ANOVA with a η_p^2 to assess the effect size of each factor under study. For each repeated measures ANOVA a *Tukey's HSD* post hoc pair-wise comparison or multiple comparisons test was employed. In *Experiment 1*, *Experiment 2*, *Experiment 3*, and *Experiment 3 + female EPN rats* as an add-on (see below) the factors were: *Test Condition* (SD, CD, CD-Rev, ID, ID-Rev, ED, ED-Rev), *Treatment* (Control vs. PERI or Control, PERI, and EPN),

and *Sex* (Male and Female) as well as interactions between these factors on the dependent measure TTC. These factors were used to assess differences in rat weight as well. A *Student's t-test* was used to assess Treatment differences (Control, PERI, and EPN) on BLLs for the dams and pups. Significance levels were set at $\alpha = 0.05$ and confidence interval (CI) of 95%. Data are presented as the mean $\pm$ SEM for rat body weights, BLLs, and all ASST behavioral data described herein.

3.0 Results

3.1. Body Weight Analysis

Prior to randomly assigning rats to the ASST food restriction protocol, there were no significant within *Treatment* group differences in weights of males or females when compared to the Control rats. There were no significant between *Treatment* group differences in weight as a function of Pb-exposure or developmental time-period of exposure. The rat weights (mean $\pm$ SEM) at the time of testing were as follows: Control males = 270.33 ± 7.37 g, EPN males = 265.50 ± 11.25 g, PERI males = 251.00 ± 12.36 g, Control females = 229.68 ± 3.94 g, EPN females = 232.83 ± 2.82 g, and PERI females = 238.83 ± 3.86 g. However, an *ANOVA* revealed a significant effect of *Sex* on body weight, $F_{(1)} = 17.630$, $p < 0.001^{***}$, $\eta_p^2 = 0.982$, but there was no significant interaction between *Treatment X Sex* on body weight.

3.2 Blood Lead Levels

The mean BLLs for the Control dams and pups were less than the lowest detectable limit of < 3.30 $\mu\text{g/dL}$ (Table 2). In contrast, PERI dams had (mean $\pm$ SEM) BLLs of 12.22 ± 0.22 $\mu\text{g/dL}$, while the EPN dams had BLLs of 4.80 ± 0.13 $\mu\text{g/dL}$. The PND 14 PERI males had (mean $\pm$ SEM) BLLs of 5.65 ± 0.13 $\mu\text{g/dL}$, while the EPN males had BLLs of 5.95 ± 0.19 $\mu\text{g/dL}$ (Table 2). The PND 14 PERI females had (mean $\pm$ SEM) BLLs of 4.38 ± 0.15 $\mu\text{g/dL}$, while the EPN females had BLLs of 5.38 ± 0.06 $\mu\text{g/dL}$ (Table 2). An *ANOVA* revealed a significant effect of *Treatment*, $F_{(2)} = 231.150$, $p < 0.001^{***}$, $\eta_p^2 = 0.955$, whereby both EPN and PERI rats had BLLs higher than Control rats. Moreover, a significant effect of *Sex* was observed, $F_{(1)} = 45.852$, $p < 0.001^{***}$, $\eta_p^2 = 0.676$, as well as a significant interaction between *Treatment X Sex*, $F_{(2,1)} = 15.449$, $p < 0.001^{***}$, $\eta_p^2 = 0.584$. The BLLs collected from the rats tested on the ASST and measured at the end of the study (PND 90–120) were below the detectable limits for the LeadCare II Blood Care Analyzer system (data not shown).

3.3 Experiment 1: Control Male vs. Control Female ASST Learning Outcomes

In *Experiment 1*, Control male and Control female rats were assessed to determine whether there were any differences in ASST performance as a function of *Sex*. Control male and Control female rats performed similarly on TTC outcomes. The Control TTC within-subjects factor data revealed a significant effect of *Test Condition*, $F_{(6,60)} = 4.653$, $p < 0.001^{***}$, $\eta_p^2 = 0.318$, but lacked a significant *Test Condition X Sex* interaction $F_{(6,1,60)} = 2.072$, $p = 0.070$ n/s (Fig. 1). For the between-subjects factors on TTC data, Control male

and Control female rats showed no significant effect of *Sex*, $F_{(1,10)} = 1.086$, $p = 0.322$ n/s (Fig. 1). The pairwise comparisons for *Sex* revealed no significant differences ($p = 0.322$ n/s) (Fig. 1) showing that Control non-Pb-exposed rats performed similarly in the ASST irrespective of *Sex*.

3.4 Male/Female ASST Learning Deficits in Response to Developmental Pb-Exposures

During the initial ASST training, dependent upon the developmental time-period of Pb-exposure, rats showed distinct behavioral differences in their abilities to learn simple discriminations (SD) for both odor (OD) and medium (MD) stimuli. These SD and OD discriminations are prerequisites for advancing to the ASST testing stages. Notably, the male EPN rats had significantly more difficulty learning the OD than the MD as evidenced by an increased TTC number, and compared to Control and PERI male rats (Fig. 2 A). Thus, male EPN rats had difficulties in learning associations that required odor sensory processing to guide food reward detection in the ASST. These findings were confirmed and the OD deficits in male EPN rats carried over into the SD stage of the ASST. During the SD stage (*i.e.*, the first stage of the ASST) all EPN male rats were unable to follow the OD stimuli (*i.e.*, had 6-consecutive errors over three independent test sessions). Therefore, they were excluded from the ASST testing phase of the study. This odor specific associative learning deficit was observed only in male EPN rats, and was not exhibited by female EPN rats (Fig. 2 B). Control, PERI, and EPN female rats showed no differences in training outcomes for both the OD and MD conditions on the TTC measure (Fig. 2 A & B).

In order to determine whether this odor specific associative learning phenomenon was a motivational and/or reinforcer satiation issue, a modified food restriction schedule (*i.e.*, reduction from 4 pellets to 2 pellets) was implemented for one day. The next day, male EPN rats were re-tested and reinforcers were increased in quantity (*i.e.*, increased from half a Froot Loop to 1 whole Froot Loop). Neither manipulation improved the male EPN rat's ability to complete the SD stage of the ASST (data not shown). These male EPN rats were not satiated to the reinforcer, as they would freely consume Froot Loops offered to them in their home cage following the cessation of testing for the day. Further, a separate assessment of repeated SDs to increasing amounts of odor (*i.e.*, 20 μ L, 50 μ L, and 100 μ L) did not improve the male EPN rat's odor associative learning ability within the training phase of the ASST (data not shown). In order to assess the effects of developmental Pb-exposure on the rats ASST performance as a function of sex, two additional experiments were conducted. In *Experiment 2*, the male Control and PERI rats were compared, where as in *Experiment 3*, the female Control and PERI rats were compared to assess the effects of developmental Pb-exposure. Then, an analysis of the male and female Control and PERI rats were conducted to determine any effects of sex and any interactions with developmental Pb-exposure. Lastly, *Experiment 3* included an add-on analysis to evaluate the female EPN rats ASST performance since, unlike male EPN rats, they were able to complete the test and their performance could be evaluated.

3.5 Experiment 2: Male Control vs. Male PERI ASST Performance

First, an ANOVA was conducted for the Control and PERI males ASST performance and with the following factors as main effects: *Test Condition* and *Treatment*, along with any

interactions between *Test Condition X Treatment* (Fig. 3 A). The TTC within-subjects factor data revealed no significant effect of *Test Condition*, $F_{(6,60)} = 1.203$, $p = 0.317$ n/s, nor a significant *Test Condition X Treatment* interaction, $F_{(6,1,60)} = 1.193$, $p = 0.322$ n/s (Fig. 3 A). Notably, the TTC between-subjects factor data revealed a significant *Treatment* effect $F_{(1,10)} = 53.320$, $p < 0.001^{###}$, $\eta_p^2 = 0.842$ (Fig. 3 A). The pairwise comparisons for *Test Condition* revealed a trend approaching significance in the SD vs. CD stages ($p = 0.56$). Thus, the data showed that the male PERI rats had deficits across their ASST performance as a function of the Pb-exposure (Fig. 3 A).

3.6 Experiment 3: Female Control vs. Female PERI ASST Performance

Next, identical to the male rats, an *ANOVA* was conducted for the Control and PERI females ASST performance using the following factors as main effects: *Test Condition* and *Treatment*, along with any interactions between *Test Condition X Treatment* (Fig. 3 B). The TTC within-subjects factor data revealed a significant effect of *Test Condition*, $F_{(6,60)} = 10.344$, $p < 0.001^{***}$, $\eta_p^2 = 0.508$, and a significant *Test Condition X Treatment* interaction $F_{(6,1,60)} = 3.218$, $p < 0.01^{\dagger\dagger}$, $\eta_p^2 = 0.842$ (Fig. 3 B). Notably, the TTC between-subjects factor data revealed a significant *Treatment* effect $F_{(1,10)} = 4.925$, $p < 0.05^{\#}$, $\eta_p^2 = 0.330$ (Fig. 3 B). The pairwise comparisons for *Test Condition* revealed a significant difference between the CD vs. CD-Rev stages ($p < 0.001^{***}$), ID vs. ID-Rev ($p < 0.001^{***}$), and ED vs. ED-Rev ($p < 0.001^{***}$). The pairwise comparisons for *Test Condition X Treatment* revealed the following significant interactions: CD vs. CD-Rev ($p < 0.001^{\dagger\dagger\dagger}$) and ED vs. ED-Rev ($p < 0.01^{\dagger\dagger}$). Thus, the data showed that female PERI rats had deficits in forming an attention-set and shifting from one attention-set to another across their ASST performance as a function of the Pb-exposure (Fig. 3 B). Further, female PERI rats had more ASST deficits than male PERI rats, suggesting a sex dependent effect that may interact with developmental Pb-exposure.

In order to test for this phenomenon, another *ANOVA* was conducted to evaluate the main effects of *Sex*, *Treatment*, and *Test Condition* and any interactions of *Sex X Treatment X Test Condition* between the Control and PERI male and female rats. The TTC within-subjects factor data revealed a significant effect of *Test Condition*, $F_{(6,120)} = 6.642$, $p < 0.001^{***}$, $\eta_p^2 = 0.249$, a significant interaction between *Test Condition X Sex*, $F_{(6,1,120)} = 3.332$, $p < 0.01^{\dagger\dagger}$, $\eta_p^2 = 0.143$, and a significant *Test Condition X Sex X Treatment* interaction, $F_{(6,1,1,120)} = 2.709$, $p < 0.01^{\dagger\dagger}$, $\eta_p^2 = 0.119$. Lastly, the TTC between-subjects factor data revealed a significant *Treatment* effect $F_{(1,20)} = 20.113$, $p < 0.001^{###}$, $\eta_p^2 = 0.501$. Taken together, these results confirmed that for PERI rats there was a significant main effect *Sex* and *Treatment* and interaction between *Sex X Treatment*, whereby females exhibited more ASST deficits than males.

3.7 Female EPN vs. Female PERI ASST Performance

In order to assess whether there are any differences in female rats ASST performance as a function of developmental time-period of exposure, the female EPN rats were added-on to

the analysis from *Experiment 3*. This analysis was restricted to females only, as the male EPN rats were excluded from the ASST testing. To frame the data in this context, Figure 4 illustrates the comparisons of the Control and PERI male rats (**A**), the Control and PERI female rats (**B**), and the Control, PERI, and EPN female rats (**C**). An ANOVA was conducted for the Control, PERI, and EPN female rats and ASST performance was compared using the following factors as main effects: *Test Condition* and *Treatment*, along with any interactions between *Test Condition X Treatment* (Fig. 4C). The TTC within-subjects factor data revealed a significant effect of *Test Condition*, $F_{(6,162)} = 37.506$, $p < 0.001^{***}$, $\eta_p^2 = 0.581$, and a significant *Test Condition X Treatment* interaction, $F_{(6,1,162)} = 8.615$, $p < 0.001^{\dagger\dagger\dagger}$, $\eta_p^2 = 0.390$ (Fig. 4C). Notably, the TTC between-subjects factor data revealed a significant *Treatment* effect, $F_{(2,27)} = 6.403$, $p < 0.01^{\#\#}$, $\eta_p^2 = 0.322$ (Fig. 4C). The pairwise comparisons for *Test Condition* revealed a trend approaching significance in the SD vs. CD stages ($p = 0.56$). Thus, the data showed that the female PERI rats had deficits across their ASST performance compared to female EPN and Control rats (Fig. 4C). The pairwise comparisons for *Test Condition* revealed a significant difference between the CD vs. CD-Rev stages ($p < 0.001^{***}$), ID vs. ID-Rev ($p < 0.001^{***}$), and ED vs. ED-Rev ($p < 0.001^{***}$). The pairwise comparisons for *Test Condition X Treatment* revealed the following significant interactions SD vs. CD ($p < 0.05^{\dagger}$), CD vs. CD-Rev ($p < 0.001^{\dagger\dagger\dagger}$), ID vs. ID-Rev ($p < 0.01^{\dagger\dagger}$), and ED vs. ED-Rev ($p < 0.001^{\dagger\dagger\dagger}$) (Fig. 4C).

3.8 ID-ED Attentional Shift Differences Between Male and Female Rats

To evaluate the extent to which developmental Pb-exposure causes deficits in the cognitive flexibility of the rats, an analysis of the ID-ED attentional shifts was conducted and displayed as box plot comparisons for male (Fig. 5 A) and female rats (Fig. 5 B). The multiple comparisons post hoc tests from the male rats (*Experiment 2*) and the add-on female rats (*Experiment 3*) were used to assess significant differences between ID-ED shifts. The data revealed no significant *Treatment* differences between the male rats ID-ED (Fig. 5 A). In contrast, the data revealed a significant ID-ED shift *Treatment* differences in female EPN ($p < 0.001^{\#\#\#}$) and female PERI rats ($p < 0.001^{\#\#\#}$), when compared to female Control rats (Fig. 5 B). The box plots in Fig. 5 suggest that female rats when Pb-exposed have more deficits in performing ID-ED shifts than Control female rats, regardless of the developmental time-period of exposure. Further, male PERI Pb-exposed rats did not show any significant differences in their ID-ED shifts compared to Controls. Taken together, these data show that sex and the developmental time-period of Pb-exposure interact to cause significant deficits in frontoexecutive dysfunctions.

3.9 Male vs. Female ASST Re-acquisition Testing Outcomes in Response to Developmental Pb-Exposures

During the ASST, after three sequential test stages (*i.e.*, SD-CD-CD-Rev) Pb-exposed rats had difficulty in continuing task performance to advance to the subsequent stages of the ASST in a single test day. Therefore, the ASST was parsed into three distinct stages (*i.e.*, Day 1: SD→CD→CD-Rev; Day 2: CD-Re-acquisition→ID→ID-Rev; and Day 3: ID-Re-acquisition→ED→ED-Rev) with the introduction of a repeated exposure (*i.e.*, a

reacquisition probe) to the last ASST test stage from the previous day (*i.e.*, either the CD-Rev or ID-Rev) to maintain the same behavioral momentum of the ASST test. Control and PERI males showed a significant *Treatment* effect across the ASST in TTC ($p < 0.01^{##}$) (Fig. 6 A) for the ID-Reacquisition conditions, with no significant differences observed in the CD-Reacquisition condition. Alternatively, the Control, PERI, and EPN female had no significant differences in the CD- and ID-Reacquisition conditions (Fig. 6 B).

4.0 Discussion

The present study showed that between Control male and female rats there were no sex-related differences in ASST training or test performance. However, following developmental Pb-exposures in age- and weight-matched rats with EPN or PERI Pb-exposures, sex-based differences in ASST performance emerged. Thus, the developmental time-period of Pb-exposure (*i.e.*, EPN or PERI), produced different patterns of frontoexecutive dysfunction. Male EPN rats were unable to consistently follow the OD, and as a result, had substantial difficulty in forming the necessary associations to complete the SD and lacked the ability to advance to and beyond the CD stage of the ASST. In contrast, male PERI rats were able to form the required associations in order to complete the ASST, but showed more difficulty in the TTC for the SD, ID, and ID-Rev stages when compared to Control males. This suggests that the PERI males have initial learning delays and exhibit difficulty with forming an attention-set, but could still form an attentional set. The PERI males exhibited more perseverative behaviors compared to Controls (*i.e.*, making repeated incorrect within-test condition daily session responses to the irrelevant stimulus without attempting to explore the other stimulus pairing) during the SD, ID, and ID-Rev discovery trials. These perseverative, impulsive, and inflexible behaviors may have contributed to the male PERI rats delays in learning the SD and difficulty in forming an attentional-set within the ID and ID-Rev stages.

Interestingly, unlike EPN male rats, EPN female rats were able to follow the OD and subsequently completed the SD and CD stages, as well as all other stages of the ASST. This suggests that in males, early postnatal Pb-exposure may directly interfere with the development of the olfactory bulb's ability to transmit sensory input during experience-dependent learning, which is contemporaneous with the developmental maturation of the rat's olfactory bulb neural circuitry over the first 3-postnatal weeks (Guthrie & Gall, 2003). Why this developmental Pb-insult would only effect males and not females, remains to be elucidated. However, it may suggest that estrogen in females may be neuroprotective to the olfactory bulb and other orbital frontal circuits, whereas testosterone may be less neuroprotective (Gold & Voskuhl, 2009). There is evidence that Pb-exposure can disrupt the neuroendocrine system within the CNS in *Drosophila* (Hirsch, Possidente & Possidente, 2010); in particular altering the duration of neural development, yet these findings require corroborating evidence in rodents. Furthermore, developmental studies have characterized that the rat's accessory olfactory bulb volume increases by 600% during the 3-postnatal weeks following birth (Rosselli-Austin, Hamilton, & Williams, 1987) and Pb-exposure during this time-period may disrupt label-line coding, connectivity, maturation, and olfactory sensory processing, specifically in males. The reasons for this sex-specific effect observed in the present study on olfactory functioning/maturation is unknown at this time. One would expect that the PERI treated rats would have greater frontoexecutive dysfunction

due to the longer time of Pb-exposure and exposure during critical early fetal development, compared to EPN rats. However, at least for males, EPN treated rats had significantly more Pb-induced behavioral deficits suggesting that the postnatal proliferating olfactory neurons may be particularly vulnerable and their disruption may underlie the sex-based ASST behavioral deficits observed. Since PERI rats were less affected by Pb-exposure in comparison to EPN rats, early and prolonged Pb-exposure may cause unique neurodevelopmental vulnerabilities as well as compensatory mechanisms during different developmental time-periods.

Alternatively, the early postnatal time-period in the rat's neurodevelopment may be particularly sensitive to environmental neurotoxicants during the same time-period when the developing brain is particularly sensitive to experience-dependent learning (*i.e.*, sensory stimulation that further guides the development of the brain in postnatal life). Furthermore, this early postnatal time-period is contemporaneous with the GABA-shift in the first 3-postnatal weeks of the rat's life (Ben-Ari, 2012; Ben-Ari et al., 2006) and the developmental pattern of the genes that regulate that GABA-shift have been shown to be altered by Pb-exposure (Neuwirth et al., 2018; Neuwirth, 2014). These observations are consistent with reports that suggest developmental Pb-exposure may disrupt early neurodevelopmental excitation-inhibition balancing mechanisms that may lead to persistent life-long disruptions in inhibitory regulatory mechanisms that contribute to proper frontoexecutive and learning functions (Neuwirth et al., 2018; Neuwirth et al., 2017; Neuwirth, 2014). These observations suggest that the rat's ASST behavioral performance may rely more heavily upon sensory function than what is currently discussed in both the neurotoxicology and the developmental behavioral neuroscience literature. Future research is needed to explore how the olfactory system develops in response to different developmental time-periods of Pb-exposure.

In contrast to male EPN rats, Female EPN rats showed a delay in learning the ID and significant difficulty in learning the ED and ED-Rev (*i.e.*, with a greater ID-ED shift), compared to the Control females. In contrast, female PERI rats exhibited an improved ability to learn the SD and ID-Rev when compared to Control females, while also exhibiting significant learning difficulties at the ED and ED-Rev conditions compared to female EPN rats. The female PERI rats in this study exhibited persistent responding to a previously correct dimension (*i.e.*, OD or MD) with perseverative qualities (*i.e.*, fixations on a single dimension), which could in part explain the improved SD and ID-Rev condition performance. Female PERI rats continued to exhibit perseverative behaviors to the previously correct dimension, which increased their TTC measures. This observation may be perhaps related to a combination of reduced inhibitory control, impulsive responding, and/or cognitive rigidity, as evidenced by their performance at the ED and ED-Rev conditions and difficulty in ID-ED shifting. Notably, female EPN rats did not show this same behavioral phenotype during the ASST, suggesting that the increased difficulty in learning the ID-Rev, ED and ED-Rev along with increased difficulty for ID-ED shifting may be due at least in part to an associative learning deficit rather than simply cognitive inflexibility or reduced inhibitory control.

The current findings in the female PERI rats are consistent with reports from Hilson & Strupp (1997) describing similar phenomena in odor-based extradimensional shift response

patterns in female Long Evans rats following developmental Pb-exposure. During the reacquisition probe stages for the CD-ReAcq and ID-ReAcq, female rats showed no differences in reacquisition learning regardless of developmental Pb-exposure, whereas male PERI rats showed a reacquisition deficit when compared to Control rats in the ID-ReAcq stage. Taken together, these findings suggest that developmental Pb-exposure may result in sex-based vulnerabilities for frontoexecutive dysfunction with different cognitive phenotypes that can be sensitively measured by the ASST in rodent models of Pb poisoning. The present study findings further suggest that rats developmentally exposed to Pb provide both construct and face validity for modeling frontoexecutive dysfunction (Roy et al., 2008) and deficits detected with neuropsychological testing (Lidsky & Schneider, 2006, Lidsky & Schneider, 2003) reported in Pb-exposed children.

The ASST has previously been shown to be sensitive in detecting the effects of specific chemical lesions of the posterior parietal cortex (Fox, Barense & Baxter, 2003) and similarly, Pb-exposure has been shown to disrupt olfactory-dependent reversal learning of extradimensional shift in tests other than the ASST (Hilson & Strupp, 1997). Data from the present study is consistent with reports identifying Pb-induced perseverative responding in delayed spatial alternation tasks (Rice & Gilbert, 1990a; Rice & Karpinski, 1998), reversal-learning tests (Rice & Gilbert, 1990b; Rice, 1992; Rabe, French, Sinha & Fersko, 1985; Sobotka et al., 1975), and repeated acquisition learning trials (Cohn & Cory-Slechta, 1993). However, caution is advised when interpreting some of these previous data since sex and developmental time-period of Pb-exposure were not examined in any of these earlier reports. For example, the work of Rice et al. (1992; 1990a; 1990b; 1988) on primates was restricted to examining both early and later postnatal differences in frontoexecutive functions, whereas more recent reports have also shown frontoexecutive decline and Alzheimer's-like neuropathological symptoms associated with early postnatal Pb-exposures (Wu et al., 2008). Since, primate studies are limited to postnatal exposures, although this model system is extremely valuable, it is equally limited as our full understanding of frontoexecutive dysfunctions induced by neurodevelopmental perinatal and postnatal Pb-exposures require adjunct rodent models (Varma, Sobolewski, Cory-Slechta, & Schneider, 2017; Verma and Schneider, 2017; Rice, 1992; Rice and Gilbert, 1990a; Rice and Gilbert, 1990b; Rice and Karpinski, 1988), and only a single study examined similar frontoexecutive behaviors in female rodents (Hilson and Strupp, 1997). Thus, the present study is the first to assess the effects of Pb-exposure on frontoexecutive functions, while evaluating both sexes, and taking into consideration different developmental time-periods (*i.e.*, PERI and EPN).

Frontoexecutive functioning underlies many important processes that maintain and produce goal-directed behaviors, action-outcome associations, shifts, stops, and value-based contingencies, choice decision making, choice impulsivity, and choice delayed reinforcement learning outcomes (for review see Dalley, Cardinal & Robbins, 2004). Further, the prefrontal cortex not only regulates these attentional processes, but is also responsible for regulating emotional responses through attentional and representational processes between the orbital frontal cortex and the amygdala (Holland & Gallagher, 1999) such as fear (Gilmartin, Balderston & Helmstetter, 2014) extinction of fear (Quirk, Garcia & González-Lima, 2006) and of extinction learning from non-aversive stimuli (Gökhan, Neuwirth & Meehan, 2017), while also ascribing emotional valence to sensory stimuli

(Hilson & Strupp, 1997). Therefore, rodent models of frontoexecutive (dys)function have become an increasingly important behavioral neuroscience translational tool (Brown & Bowman, 2002), either through animal models that simulate neurological double-dissociations (Bissonette et al., 2008) or single-dissociative neurotoxic lesion studies (Fox, Barense & Baxxter, 2003; Wallace, Kolb & Whinshaw, 2002). Moreover, since the preclinical Pb neurotoxicity field has focused more on hippocampal-dependent learning and memory and less on frontoexecutive function, the current study provides new information adding to our understanding of how low Pb-exposure influences frontoexecutive functions across an organism's lifespan.

Since developmental Pb-exposure has been shown to disrupt the development of the GABAergic system (Neuwirth et al., 2018; Neuwirth, 2014) resulting in aberrations in behavioral inhibitory control (Neuwirth et al., 2017), the current findings suggest the possibility that similar GABAergic reductions in inhibitory frontoexecutive functioning may be influencing learning and decision making processes assessed in the ASST. More work in this area is needed to understand the extent to which sex-specific dysexecutive functions related to GABAergic and non-GABAergic-mediated mechanisms may be involved in response to developmental Pb-exposure.

Reports on the neural structures underlying the set-shifting behaviors in rats have directed much focus to the medial prefrontal cortex and the anterior cingulate cortex (Bissonette et al., 2013). Excitotoxic lesions to the orbital prefrontal cortex has been shown to disrupt reversal learning, whereas medial prefrontal lesions has been shown to impair the ED condition of the ASST providing a dissociation of behaviors and prefrontal cortical brain regions regulating frontoexecutive functions (Bissonette et al., 2008). Based on the present data, it is conceivable that Pb-exposure may disrupt the neurochemical signaling within and perhaps between the orbital frontal cortex and the medial prefrontal cortex in PERI and EPN treated rats interfering with performance at the reversal and ED stages. Consistent with the present data, cortico-striatal GABAergic tone when reduced can significantly impair reversal learning in the ASST (Martins et al., 2011). Further, the anterior cingulate cortex has also been shown to play a significant role in processing the meaning of associative cues within a given perceptual dimension (*i.e.*, discriminating between two digging mediums within the ID stage), whereas the medial prefrontal cortex is necessary for switching between dimensions (*i.e.*, when moving from the ID to the ED stages of the ASST) (Ng et al., 2007). Thus, it is also possible that developmental Pb-exposure may disrupt the anterior cingulate cortex in male PERI and female EPN rats, while female EPN and PERI rats may have additional aberrant neural circuitry in the medial prefrontal cortex as they have significant difficulty switching dimensions for the ID to ED stages of the ASST.

5.0 Conclusion

The present study is the first report to show that animal models of childhood Pb poisoning produces frontoexecutive dysfunctions commensurate with the clinical literature. Further, the Pb-exposed rats in the present study exhibited different frontoexecutive dysfunctions dependent upon sex-based vulnerabilities to Pb and the developmental time-period of Pb-exposure. Moreover, this study concurrently examined both sexes and different time-periods

of developmental Pb-exposure on frontoexecutive dysfunctions that persisted across the lifespan, which is consistent with an animal model of intellectual disability in response to an early exposure to an environmental neurotoxicant. Thus, the present study shows that the ASST in rats can provide construct, face, and predictive validity for Pb-induced frontoexecutive deficits. Moreover, the present study's findings show that examining frontoexecutive (dys)functions, in addition to hippocampal-dependent learning, is important for understanding the bases of the neuropsychological deficits that occur consequent to developmental Pb-exposure.

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HIGHLIGHTS:

- Control Male & Female rats do not differ in ASST performance.
- Developmental Pb-exposure causes sex-based differences in ASST performance.
- Developmental time-period of Pb-exposure causes differences in ASST performance.
- EPN and PERI Pb-exposures cause different sex-based ASST learning deficits.
- EPN males could not complete the ASST, while PERI males could with delays.
- EPN and PERI females required twice as many trials to complete the ASST.
- The ASST can identify frontoexecutive dysfunctions in Pb-exposed rats.

Figure 1.

Male (white bars) and female (grey stippled bars) Control rats ASST performance for trials-to-criterion (TTC). Control male and female rats were able to form attention sets evidenced by solving the ID stages with less trials and errors than the ED stages. Control male and female ASST test performance was not significantly different from one another, yet a trend was observed that female rats tend to require more trials in learning the ID-Rev stage. Data are presented as the mean $\pm$ SEM, with $\alpha = 0.05$, and CI of 95%.

Figure 2.

Control, PERI, and EPN male (**A-solid bars**) and female (**B-stippled bars**) trials-to-criterion (TTC) in ASST training when learning simple discriminations of odor (OD) and digging medium (MD). EPN exposure caused a learning deficit only in male rats when associating odors during training evidenced by increased the TTC ($p < 0.01^{##}$) in the OD. Legend: ([#]) = *Treatment Effect*. Data are presented as the mean $\pm$ SEM, with $\alpha = 0.05$, and CI of 95%.

Figure 3.

Control and PERI male rats **(A)** and Control and PERI female rats **(B)** trials-to-criterion (TTC) in the ASST. PERI male rats showed delays in learning the SD, ID and ID-Rev conditions, yet solved the ED and ED-Rev stages similar to Control males **(A)**. This data suggests male PERI rats has deficits in forming attention-sets. In contrast, PERI female rats **(B)** were able to form attention-sets like Control female rats, but had significant learning delays in the ED and ED-Rev conditions. This data suggest that PERI females have different frontoexecutive dysfunctions than PERI males despite the same developmental Pb-exposure, and, females required nearly twice the amount of TTC to complete the ED-ED-Rev conditions than males. Legend: (*) = *Test Condition* Effect; (#) = *Treatment* Effect; (†) = *Test Condition X Treatment* Interaction. Data are presented as the mean $\pm$ SEM, with $\alpha = 0.05$, and CI of 95%.

Figure 4.

illustrates the comparisons of the Control and PERI male rats (A) in *Experiment 2*, the Control and PERI female rats (B) in *Experiment 3*, and the Control, PERI, and added on EPN female rats (C) in *Experiment 3 + EPN* female rats. Male PERI rats (A) exhibit different frontoexecutive dysfunctions than female PERI rats (B), when compared to Control male and female rats. Further, female PERI rats exhibit different frontoexecutive dysfunctions than female EPN rats, when compared to Control female rats (C). The data suggest that dependent upon the developmental time-period of Pb-exposure and sex that different types of frontoexecutive dysfunctions may occur within the prefrontal cortex in rats that persist across the lifespan. Legend: (*) = *Test Condition* Effect; (#) = *Treatment* Effect; (†) = *Test Condition X Treatment* Interaction. Data are presented as the mean for each test condition of the ASST.

Figure 5.

illustrates the Control (white) and PERI (dark grey) male rats ID-ED abilities from *Experiment 2* (A) and the Control (white), PERI (dark grey), and EPN (light grey) female rats ID-ED shift abilities from *Experiment 3 + EPN female rats add-on* (B). The data revealed no significant differences in Control and PERI male rats ID-ED shifting abilities. In contrast, the data revealed a significant ID-ED shift difference in female EPN ($p < 0.001^{***}$) and female PERI rats ($p < 0.001^{***}$), when compared to female Control rats (B). The data suggest that females rats when Pb-exposed had more deficits in ID-ED shifts than Control female rats, regardless of the developmental time-period of exposure. In addition, Pb-exposed female rats have more deficits in ID-ED shifts than male Pb-exposed rats. Thus, female Pb-exposed rats exhibit sex-based differences in ID-ED frontoexecutive dysfunctions. Legend: (#) = *Treatment Effect*. Data are presented as box plots with the solid black line as the median, the lower line, lower box, upper box, and upper line serving as quartiles 1, 2, 3, and 4, respectively.

Figure 6.

Control and PERI male rats (A) and Control, PERI, and EPN female rats (B) ASST re-acquisition outcomes. Control and PERI males revealed a significant *Treatment* effect across the CD and ID re-acquisition conditions ($p < 0.01^{##}$) (A). In contrast, the Control, PERI, and EPN females showed no significant differences in the CD and ID re-acquisition conditions (B). The data suggest that PERI male rats have difficulty with learning re-acquiring prior learned information after already having learned an attention-set, yet PERI female rats do not have such problems. Legend: (#) = *Treatment* Effect. Data are presented as the mean $\pm$ SEM, with $\alpha = 0.05$, and CI of 95%.

Table 1.

The odor exemplar pairing used in the attention set-shifting task

Odors			Digging Media	
Pairings				
Training	O1-Cumin	O2-Paprika	M1-Shredded Paper	M2-Polystyrene
Testing				
Pairing 1	O3 – White Thyme	O4 – Texas Cedar Wood	M3 – Small Beads	M4 – Small Gravel
Pairing 2	O5 – Clove Buds	O6 - Rosemary	M5 – Fine Wood Shavings	M6 – Large Wood Shavings
Pairing 3	O7 - Spearmint	O8 - Cinnamon	M7 – Dirt with Wood Shavings	M8 - Mulch

Abbreviations are defined as follows: Pairing 1 comprised the **(SD)** = Simple Discrimination, **(CD)** = Compound Discrimination, and the **(CD-Rev)** = Compound Discrimination Reversal stages; Pairing 2 comprised the **(CD-Reacq)** = Compound Discrimination Reacquisition, **(ID)** = Intra-dimensional Shift, and the **(ID-Rev)** = Intra-dimensional Reversal stages; and Pairing 3 comprised the **(ID-Reacq)** = Intra-dimensional Shift Reacquisition, **(ED)** = Extra-dimensional shift, and the **(ED-Rev)** = Extra-dimensional Shift Reversal stages.

Table 2.

Blood lead levels (BLLs) measured at postnatal day (PND) 14 in pups and Dams at weaning

Conditions	Control	Perinatal (PERI)	Early Postnatal (EPN)
Dams	< 3.33	12.22 ± 0.22***	4.80 ± 0.13*** ###
Male PND 14 pups	< 3.33	5.65 ± 0.13***	5.95 ± 0.19*** ###
Female PND 14 pups	< 3.33	4.38 ± 0.15***	5.38 ± 0.06*** ###

The data are presented as µg/dL mean blood lead levels and ± SEM. Sample size $n = 4$ for male pups, $n = 5$ for female pups and Dams per group. Significant differences are noted between Control and either PERI or EPN $p < 0.001$ ***, as well as, PERI and EPN $p < 0.001$ ###.