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Evaluation of Environmental Enrichment and Pup Exposure on Maternal Behavior Sensitization Latencies in Virgin Female Rats

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ABSTRACT

Adult, virgin female rats do not spontaneously display maternal behavior, but can be "sensitized" to display such behaviors through 5-7 days of continuous exposure to foster pups. The purpose of this study was to decrease the anxiety of the animals through environmental enrichment, and to determine if the resulting reduced stress would stimulate faster maternal behavior onset, therefore reducing the number of animals needed in the maternal behavior sensitization testing paradigm. However, animals in an enriched environment displayed longer maternal behavior onset latencies.

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ACKNOWLEDGEMENTS

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1. BACKGROUND

Extensive studies of animal behavior have revealed an intensely complex and sophisticated system in which animals thrive. This system relies on the animals' dependence on, and cooperation with, the particular environment in which they exist. Several species of animals have demonstrated intricate systems through which they survive, some of which are too complex to be understood by scientists in the near future. A particular species of animal on which much behavioral research has been and continues to be performed is the rat. Research using these animals has led to several important milestones in the scientific realm.

1.1 Maternal Behavior

Maternal behavior in the rat is defined as the specific behaviors that the mother rat performs in order to allow for successful nurturing and rearing of her young. These behaviors are under strict neuroendocrine and neurochemical control, and several research studies have provided a wealth of information related to maternal behavior systems in the rat (Mann, 1993).

1.1.1. Behavioral Characteristics

Rat maternal behaviors typically include nest building, retrieval of the rat pups, grouping of the rat pups in the nest, crouching over the pups in the nest, and anogenital licking of the pups (Mann, 1993). Female rats normally display this maternal behavior at parturition, at which time the behavior is strongly evident and immediate. Initiation of this behavior is thought to result from endocrine changes that occur during pregnancy (Numan & Insel, 2003).

1.1.2. Hormonal Control of Rat Maternal Behavior

Several experiments have shown that hormones play a major role in the onset of maternal behavior after parturition, and the hormones that play major roles are estradiol, progesterone, prolactin, and placental lactogens. **Figure 1** shows the various changes in peripheral plasma levels of these hormonal constituents throughout the pregnancy period. **Figure 1A** indicates that estradiol levels are low at the beginning of pregnancy, and rise sharply around day 15. In contrast, progesterone levels are high at the beginning of pregnancy and fall sharply around day 15. Therefore, the progesterone-to-estradiol ratio is reversed shortly before parturition. Prolactin (secreted from the anterior pituitary) and placental lactogens (secreted from the placenta) are lactogenic hormones, which function to promote mammary gland development and lactogenesis. **Figure 1B** indicates that prolactin levels are high at the beginning of pregnancy, and fall around day 11, around the time that placental lactogen levels begin to rise (Numan & Insel, 2003). Several research studies have proven that this hormone regimen is stimulatory for maternal behavior (Moltz et al., 1970; Zarrow et al., 1971).

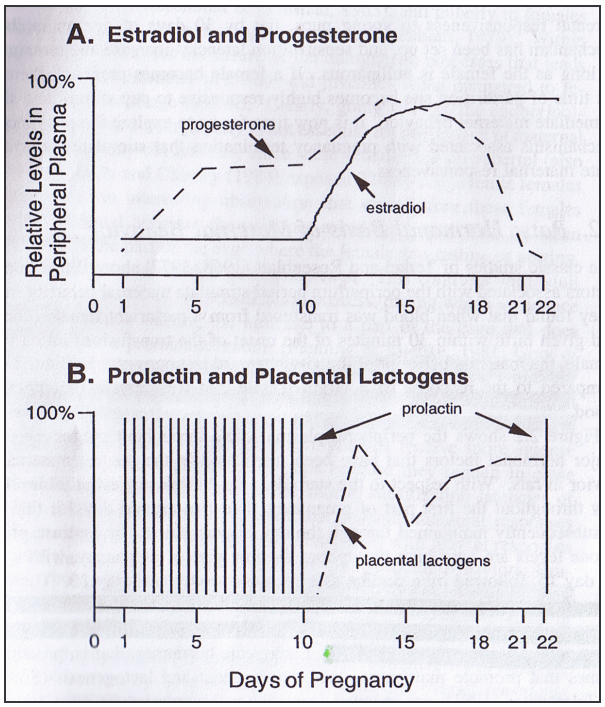

Figure 1: Relative blood plasma levels of estradiol, progesterone (**A**), prolactin, and placental lactogens (**B**) over the course of the rat's 22-day pregnancy (Numan & Insel, 2003).

1.1.3. Nulliparous Rat Maternal Sensitization Behavior

In contrast to female rats at parturition, adult nulliparous (virgin) rats do not immediately display these maternal behaviors when exposed to foster rat pups. When a foster pup is placed in the presence of an adult virgin female rat, the female will briefly

sniff the pup and then quickly back away. The female will usually then completely ignore the foster pup, but she occasionally may cannibalize the pup or bury the pup under the bedding in the cage (Rosenblatt, 1969). Although adult virgin female rats will not usually display maternal behavior immediately in the presence of foster pups, constant 24-hour exposure to foster pups will cause the amount of time spent avoiding the pups to gradually decrease. The female rat becomes tolerant to the proximity of the pups, and eventually the virgin rat will display each behavior that is characteristic of normal maternal behavior. This process of the onset of maternal behavior is called *sensitization*. The sensitization period usually has a latency of 5-7 days (Numan & Insel, 2003).

The failure of virgin adult female rats to immediately display maternal behavior in the presence of foster pups has been explained by what can be called an approach-avoidance model of the onset of maternal behavior (Rosenblatt & Mayer, 1995). This motivational model explains that maternal behavior occurs when the tendency of the female rat to interact with the pup is greater than her tendency to withdraw from the pup. Repeated exposure to foster pups brings the female closer to the threshold point at which the tendency to interact becomes greater than the tendency to withdraw. The onset of maternal behavior according to this approach-avoidance model involves the activation of internal motivational processes that increase the female's attraction to and interest in the pup, as well as the depression of internal processes that prevent maternal behavior.

1.1.4. Sensory and Neural Control of Maternal Behavior Sensitization

The latency of nulliparous adult female rats to display maternal behavior is affected largely by the different sensory systems of the rat and the brain regions to which they project. Most importantly, the olfactory system plays a major role in the regulation of the onset of maternal behavior. **Figure 2** shows a sagittal section through the rodent cranium, showing the nasal cavity

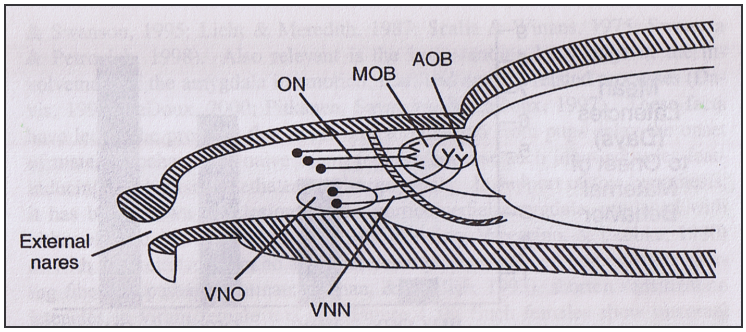

Figure 2: Rodent Cranium Sagittal Section. AOB = accessory olfactory bulb; MOB – main olfactory bulb; ON = olfactory nerve; VNN = vomeronasal nerve; VNO = vomeronasal organ (Numan & Insel, 2003).

(opening to the left) and the olfactory chemosensory apparatus (center of the diagram). The primary olfactory epithelium contains the sensory receptors of the primary olfactory nerve, which projects to the main olfactory bulb (shown dorsally in the diagram). Also located in the nasal cavity is the vomeronasal organ (shown ventrally in the diagram), whose axons also terminate in the olfactory bulb. Both of these systems are involved in the detection of pheromones, and both systems project to the amygdala, a brain regions which has been shown to be involved in emotion, fear, and anxiety-related processes (Numan & Insel, 2003). Therefore, it has been suggested that the novel olfactory stimuli from the pups initially delays the onset of maternal behavior by activating fear and anxiety processes through the amygdala. Studies have shown that lesions to the amygdala shorten the sensitization latency period in adult female rats (Del Cerro, 1998). Amygdala lesions mainly eliminate the initial avoidance of the pups, but pup stimulation is still needed over a few days to allow the overall level of maternal behavior to increase fully (Fleming et al., 1980).

1.1.5. Maternal Behavior Sensitization Testing

A standard maternal behavior sensitization paradigm is used in most maternal behavior studies. The virgin female rats, which had been previously housed doubly or triply, are singly housed. Three pups between 3 and 8 days old are then placed in the cage. The pups must not The female subject is directly observed for 15 minutes and then spot-checked at subsequent 15-minute intervals for one hour in order to record when the subject displays behaviors that are considered maternal. The pups are left in the subject's cage for 24 hours, and are removed the next day and replaced with three more freshly fed pups. This procedure continues until the female shows full signs of maternal behavior, and the animal is considered to be fully maternal on the third consecutive day of full maternal behavior (Mann, 1993).

A number of concerns have arisen with respect to the protocol for maternal behavior sensitization testing. The first of these concerns is that the single-housing that is necessary for sensitization testing has been shown to increase stress levels in the rats (Einon et al., 1981; Leshem and Sherman, 2006). It is possible that the maternal behavior sensitization latencies may be adversely affected as a result of these increased stress

levels. A second concern in the maternal behavior testing protocol involves the number of foster pups and their donors that are necessary for testing. Three 3- to 8-day old pups are used for testing per subject animal, and must be replaced with three more freshly fed pups during each subsequent 24-hour exposure period. Since pups must not be older than 8 days (older pups are able to crawl towards the female rat), and sensitization latencies can sometimes reach as many as 11 days, a significantly large number of pups and their donors are needed for the testing protocol.

1.2 Environmental Enrichment

In laboratory research using rats, environmental enrichment involves enhancing the cage conditions of the animal beyond the food, water, and bedding material that is normally provided. Studies using environmental enrichment have been used to demonstrate various changes in the neuroanatomy, physiology, and behavior of the animals as a function of the environment.

1.2.1. Physical Effects of Environmental Enrichment

Several experiments have been performed that have highlighted several physiological and behavioral effects in rats produced through environmental enrichment. Enriched and complex environments have been shown to induce hippocampal neurogenesis (Kempermann et al., 1997; Olson et al., 2006). Environmental enrichment has been shown to directly increase dendritic spine density; levels of synaptic proteins, receptors, and neutrotrophins; cortical reorganization; and learning and memory. Each of these factors has been shown to enhance levels of neurogenesis and the number of synapses per neuron (Leggio et al., 2005).

Other studies have shown environmental enrichment to alleviate rat memory impairment after experimental stroke (Dahlqvist et al., 2004). An enhanced environment increases brain plasticity and improves sensorimotor functions, improvements which eventually lead to increased functionality of memory. In addition to improved memory after stroke, environmental enrichment has also been shown to decrease the recovery time required from brain lesions (Maegele et al., 2005).

1.2.2. Hormonal Effects of Environmental Enrichment

In addition to the many effects that environmental enrichment has on physical and behavioral aspects of rats, environmental enrichment also produces significant hormonal changes in laboratory rats. Several studies have shown that exposure to stressful stimuli causes increased plasma concentrations of glucocoritcoids and catecholamines, which over time can lead to detrimental effects on the health of the animals, both mentally and physically (McEwen & Sapolsky, 1995). Studies have shown that individual housing of laboratory rats, rather than group housing, creates stress in the animals and can be considered a deprivation. Isolation of the animals causes increases in the levels of timidity and aggression in the animals (Einon et al., 1981). Because adult nulliparous rats must be singly housed for maternal behavior testing, the stress that results from isolation is a cause for concern.

Environmental enrichment has been shown to decrease stress levels in rats. In a study done by Moncek et al. (2004), levels of hormones shown to increase due to stressful stimuli (adrenocorticotropic hormone (ACTH), corticosterone, and adrenaline) were compared in rats kept in enriched environments versus standard housing. Levels of these hormones were found to be initially higher in the animals in the enriched environment, which resulted from the fact that those animals were exposed to more novel stimuli compared to the rats in standard housing, and these novel stimuli are considered stressful. However, during subsequent frequent handling of the animals, the rats in the enriched environment showed lower levels of stress hormones compared to the rats in standard housing. These results indicate that the presence of novel objects in the enriched environment causes a sensitization in the animals that decreases the amount of stress caused during exposure to new stressful stimuli. In relation to maternal behavior, it can be predicted that rats housed in an enriched environment while undergoing sensitization should show lower stress hormone levels in response to the novel stimuli of pups compared to rats in standard housing.

2. PROJECT PURPOSE

Adult, virgin rats do not normally display spontaneous maternal behavior when exposed to foster pups. However, continuous daily exposure to pups can sensitize the female in 5-7 days so that she displays maternal behavior that is almost indistinguishable from normal postpartum behavior. The time it takes for the sensitized female to become maternal may depend upon several factors, including stress and anxiety. Since studies have shown that isolating rats to single cages is stressful, sensitization latencies may be adversely affected as a consequence.

The first objective of this study was to mitigate the stressfulness of being singly housed during the sensitization period by enriching the rat's environment, and to determine if the resulting reduced stress would stimulate shorter maternal behavior onset latencies. If the females were able to develop maternal behavior more quickly due to the enriched environment, fewer foster pups (and their donors) would need to be used in subsequent maternal behavior studies.

The second objective of this study was to determine if the number of pups used to sensitize the adult females could be reduced to one. The majority of maternal behavior experiments performed with virgin rats use three 3- to 8-day old foster pups to stimulate maternal behavior. It is possible that the use of one pup may stimulate maternal behavior to the same extent as three, or a combination of using one pup in an enriched environment may stimulate shorter maternal behavior sensitization latencies. The use of one pup instead of three would substantially reduce the number of necessary foster pups and their donors. The major aim of this study was to both refine and reduce the use of animals in maternal behavior studies.

3. MATERIALS AND METHODS

3.1 Animals

Nulliparous (virgin) Sprague-Dawley female rats (225-250g; CRL:CD(SD)BR) were purchased from Charles River Laboratories, Inc. 50 rats were designated as the experimental animals. For the first week, these 50 females were individually housed in standard housing conditions, consisting of polypropylene cages (45 x 25 x 20 cm) that contained approximately 1.5 liters of medium grade wooden flakes. Food and water were available ad libitum in a light- (on 0500-1900h) and temperature- (21-25°C) controlled room.

A separate group of 30 female rats were used to generate foster pups for maternal behavior testing. These donor females were kept in a separate room from the experimental animals, since the sound and smell of pups can affect maternal behavior latencies.

All animals were maintained in accordance with the guidelines of the Division of Teaching and Research Resources at Tufts University, Cummings School of Veterinary Medicine, which follows the procedures for animal care prepared by the Committee on the Care and Use of Laboratory Animal Resources, National Research Council.

3.1.1. Experimental Groups

After the first adaptation week in the standard environment, animals were divided into two experimental groups of different experimental environments, shown in **Table 1** below:

Group #	Environment	# Rats
1	Control	25
2	Enriched	25

Table 1: Experimental Groups

The control environment consisted of normal housing conditions described above. The enriched environment included the normal housing conditions plus a red crawl ball (**Figure 3A**) and wood chunk (**Figure 3B**), ordered from Bio-Serv®. In addition, plastic dividers (2 inches high) were placed in each cage, for both the control and experimental groups. The dividers served to separate the cage into four equal quadrants, in order to

prevent the test pups from crawling towards the female during maternal behavior testing. Animals were given an additional week of adaptation in their respective environments before elevated plus maze testing and maternal behavior testing were begun. This week was necessary in order to allow adaptation to the different environments, in order to prevent any false conclusions made from the initially stressful adaptation stage on the results of maternal behavior testing.

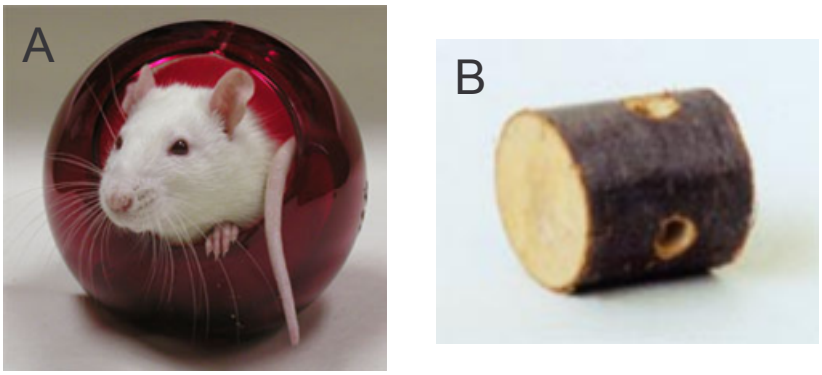

Figure 3: Bio-Serv® (A) crawl ball; (B) wood chunk. (www.bio-serv.com)

3.2 Elevated Plus Maze Testing: Pre-test

In order to asses differences in the levels of anxiety-like behaviors for the control versus enriched environments, animals were tested in a Hamilton-Kinder® automated elevated plus maze (EPM) system (Poway, CA) after the one-week environmental adaptation period. The elevated plus maze consists of two closed arms (50.2 cm × 10.8 cm) and two open arms (50.2 cm × 10.8 cm) without edges, forming a "Plus" or cross that stands 85.1 cm off the floor (see **Figure 4**). The subjects were placed in the intersection (10.8 × 10.8 cm) facing an open arm. At that point, data collection began using Microsoft compatible MotorMonitor® software (Hamilton-Kinder), and continued for 5 min. Movement through the maze was detected by 48 equally spaced photocells embedded in each arm of

Figure 4: Hamilton-Kinder® Elevated Plus Maze

the apparatus. Ethanol (70%) was used to clean the apparatus between individual test sessions. The following data were collected: overall (total) activity (number of beam breaks), distance traveled in the open and closed arms, and time spent in the open or closed arms. Increased time, and/or distance traveled in the open arms was interpreted as reflecting reduced anxiety-like behavior (Lister, 1990).

In this study, all subjects were tested on the plus maze before maternal behavior testing began (pre-test). This EPM pre-testing was performed in order to indicate differences in anxiety-like behaviors affected only by the factor of environment, and not affected by the additional stressor of pups.

3.3 Maternal Behavior Testing

After EPM pre-testing was performed, experimental animals were further sub-divided into four experimental groups based on the number of foster pups used for testing, shown in **Table 2** below:

Group #	Number of Pups	Environment	# Rats
1a	1	Control	12
1b	3	Control	13
2a	1	Enriched	13
2b	3	Enriched	12

Table 2: Experimental Groups

Maternal behavior testing began immediately following the EPM pre-test at 0900h. First, the quadrant in which the female was located was recorded, and for the environmentally enriched animals, the location of the ball and wood chunk were also recorded. Either one or three freshly fed foster pups were placed in the cage away from where the female was located. The animals were then observed directly for 15 minutes for signs of maternal behavior. The following maternal behaviors and the times at which they occurred were recorded: initial contact with the pup(s); retrieval of each individual pup into a common quadrant; grouping of the pups into a common quadrant; and crouching of the female over the pup(s). In addition, for the animals in an enriched environment, the number of contacts made with the ball or wood chunk were also recorded. The sheet used to record testing data can be seen in Appendix I.

After continuous observation for 15 minutes, the animals were then spot checked at 15 minute intervals for up to an hour. Spot checking is defined as simply recording the quadrant locations of the female, pups, ball, and wood chunk. After the spot checks, the pups were left in the cages with the females over night. The next day, again at 0900h, a spot check was again performed. The pups were then removed from the cage, and after an hour, one or three more freshly fed pups were placed in the cage with the female and the testing procedure was performed again. In the event that a female killed a test pup, it was given only one pup (in the 3-pup groups) the next day until the pup-killing stopped. The data sheet used to record the maternal behavior testing data can be seen in Appendix I.

Maternal behavior testing continued on a daily basis until the females responded maternally. Full maternal behavior is defined as retrieving, grouping, and crouching over all the test pups within the one-hour testing period (Mann, 1993). The latency, in days, to become fully maternal was calculated as the first day of two consecutive days of full maternal behavior. For example, if an animal exhibited full maternal behavior on the first and second days of testing, that female would receive a latency score of zero days.

3.4 EPM Post-test and Corticosterone Radioimmunoassay (RIA)

In order to observe differences in response to stress between the experimental groups, a corticosterone radioimmunoassay (RIA) was performed. Following maternal behavior testing, experimental animals were again tested on the EPM. This EPM post-test served to act as a stressor to the animals, so as to compare the corticosterone response to stress between experimental groups. The first 41 rats to display full maternal behavior were subjected to this post-test, and the last 9 animals were not post-tested in order to serve as a baseline control for the corticosterone assay.

Immediately following all behavioral testings, all subjects were euthanized by rapid decapitation. Trunk blood was collected into heparinized tubes, and centrifuged. Blood plasma was stored at −20 °C. Plasma corticosterone concentrations were measured by radioimmunoassay (RIA) using a commercially available kit (Diagnostics Products Corporation, Los Angeles, CA).

3.5 Statistical Analyses

Data was analyzed using parametric and nonparametric statistics. The percentage of rats maternal on each test day was analyzed using the Fisher Test for Exact Probability. Latencies in days to respond maternally were analyzed using a two-way analysis of variance (ANOVA), with environments (control versus enriched) and pup number (one versus three) as the independent factors. Elevated plus maze (EPM) data was analyzed using the *t*-test. Post-hoc comparisons were done with the Least Significant Difference test. Differences were considered significant if $P<0.05$.

4. RESULTS

4.1 Maternal Behavior Testing

The percentages of animals responding maternally on each test day are shown in **Figure 5**. The percentage of rats maternal on each test day was analyzed using the Fisher Test for Exact Probability. The number of control with one foster pup (control/1 pup) animals (closed circles) that were maternal on day 3 was significantly higher (asterisks) than all other groups (all $P<0.05$). The number of control/1 pup animals maternal on day 4 was significantly higher than either enriched group (both $P<0.05$).

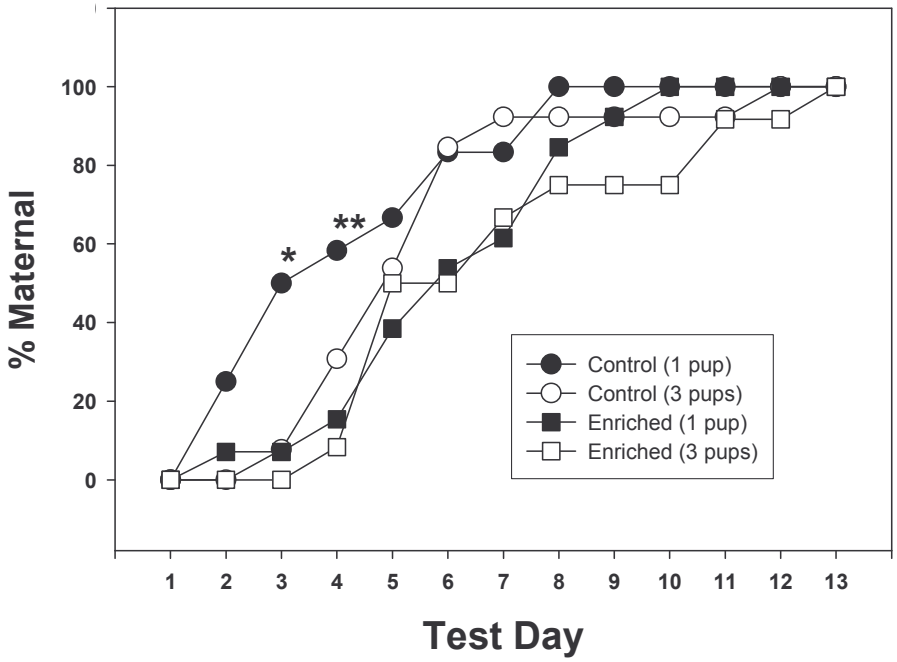

Figure 5: Percentages of animals maternal on each test day. Control (1 pup) N=12; Control (3 pups) N=13; Enriched (1 pup) N=13; Enriched (3 pups) N=12. Data analyzed by Fisher Test for Exact Probability. *Significantly different from all groups. **Significantly different from enriched groups. (all $P<0.05$)

The mean latencies (in days) to display full maternal behavior are shown in **Figure 6**. Latencies in days to respond maternally were analyzed first by two-way analysis of variance (ANOVA), with environments (control versus enriched) and pup number (one versus three) as the independent factors. There was a significant effect of treatment, with animals in the control environment (first two histobars) becoming

maternal more rapidly than animals in the enriched environment (right two histobars) (P=0.012). There was no statistically significant effect of pup number, and no significant interaction between the two factors.

The latencies in days to respond maternally were then analyzed by the Least Significant Difference Test. Animals with one pup in the control environment became fully maternal significantly faster than animals in the enriched environment with one pup (P=0.040).

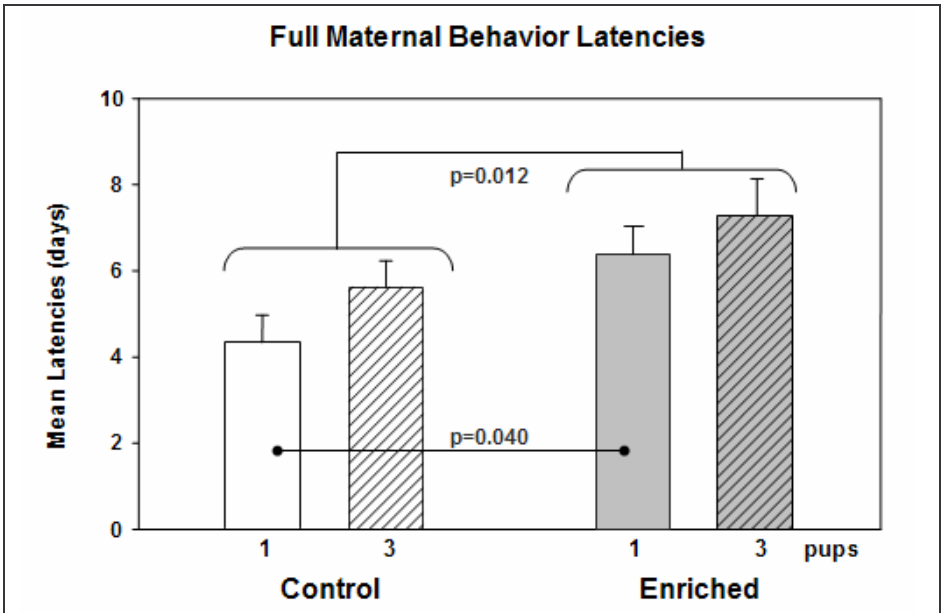

Figure 6: Mean latencies (in days) for animals to respond maternally. Control (1 pup) N=12; Control (3 pups) N=13; Enriched (1 pup) N=13; Enriched (3 pups) N=12. The data was analyzed by two way ANOVA (P=0.012) and LSD test (P=0.040).

4.2 Elevated "Plus" Maze: Pre-test

After one week of exposure to their respective environments, animals were tested on the elevated "plus" maze (EPM) before maternal behavior testing was begun. Data collected included basic movements, fine movements, and the time in, distance traveled in, and entries into the open arms, closed arms, and intersection of the plus maze. The pre-test EPM data for both basic and fine movements are shown in **Figure 7**. There is a trend towards less basic movements for the enriched group (gray histobar) ($p<0.062$, *t*-

test), and there were significantly less fine movements for the enriched group ($p<0.045$, Mann-Whitney test).

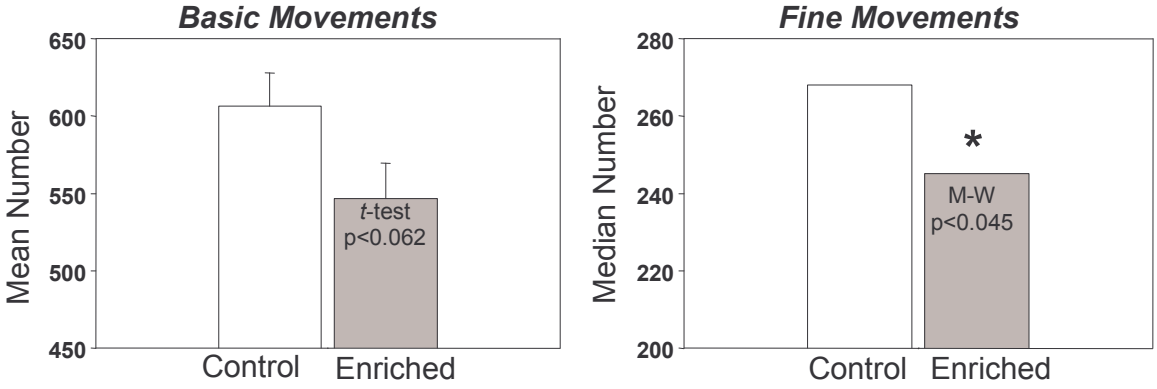

Figure 7: Pre-test EPM Basic Movements (*t*-test) and Fine Movements (Mann-Whitney test). Control N=25; Enriched N=25. *Statistically Significant

The data for movements in the open arms of the plus maze are shown in **Figure 8**. Only the movements in the open arms are shown (movements in closed arms and intersection not shown) because they are the most indicative of reduced-anxiety-like behavior. EPM data for the open arms was analyzed using the *t*-test. There was a trend towards less movement in the open arms of the maze for the enriched environment (panel A, gray histobar) compared to animals in the control environment (open histobar). Time spent and distance traveled in the open arms was decreased, but not statistically significant for the enriched animals (**Figure 8A/B**). Entries into the open arms was significantly lower for enriched animals than for control animals ($P<0.034$) (**Figure 8C**).

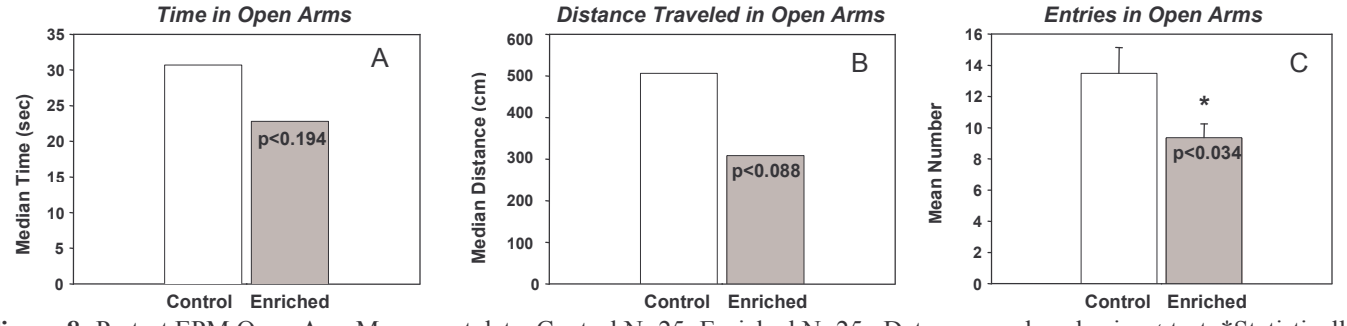

Figure 8: Pretest EPM Open Arm Movement data. Control N=25; Enriched N=25. Data was analyzed using *t*-test. *Statistically Significant

4.3 EPM Post-test and Corticosterone Radioimmunoassay (RIA)

Elevated plus maze (EPM) post-testing was used as a stressor in order to compare corticosterone stress responses among the experimental groups. After maternal behavior testing, the first 41 animals were again tested on the EPM. Post-test EPM data are shown in **Figure 9**. There were no significant effects of treatment (environment) or pup number on the movements in the open arms of the maze during the EPM post-test.

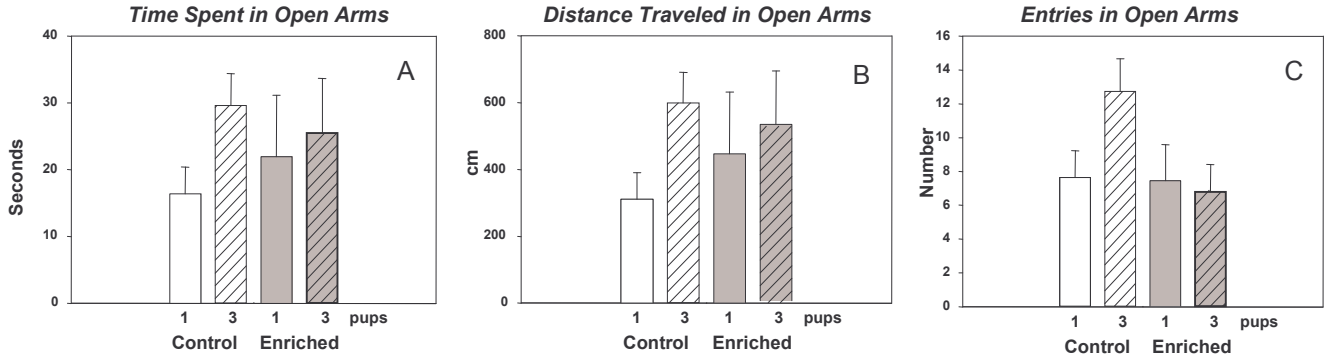

Figure 9: Post-test Elevated Plus Maze (EPM) Open Arm Movement Data. Control (1 pup) N=12; Control (3 pups) N=13; Enriched (1 pup) N=13; Enriched (3 pups) N=12. Data was analyzed using t-test.

Following EPM post-testing, plasma corticosterone levels were measured in all experimental animals. The 9 animals not subjected to an EPM post-test served as baseline values for plasma corticosterone levels. Plasma corticosterone data was analyzed by a one-way analysis of variance (ANOVA), and is shown in **Figure 10**. Plasma corticosterone levels were significantly higher for all experimental groups versus baseline (all $P<0.001$), indicating that the EPM post-testing successfully served as a stressor for the animals. However, no significant differences existed between the experimental groups.

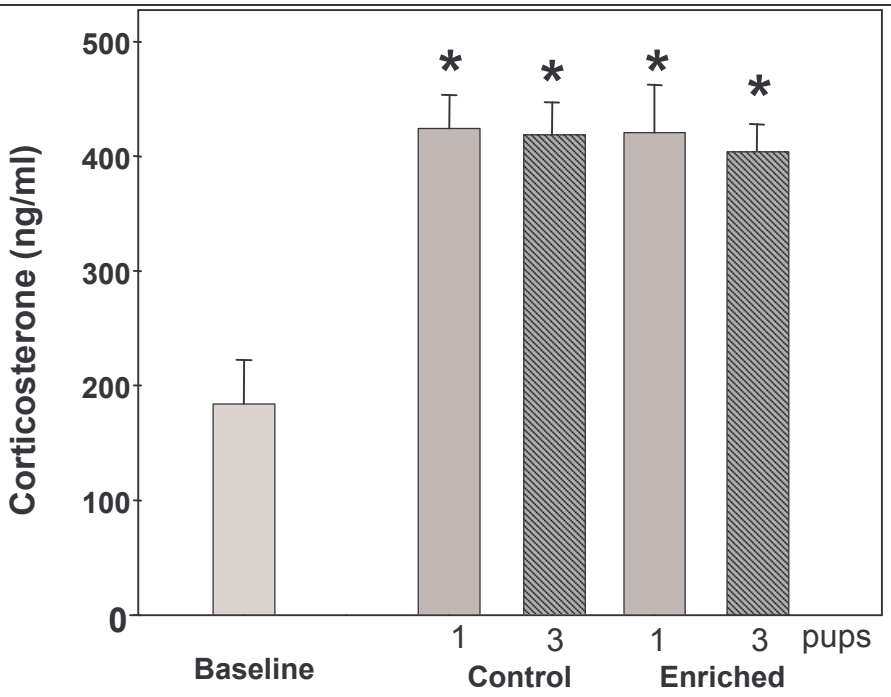

Figure 10: Plasma Corticosterone Levels. Control (1 pup) N=12; Control (3 pups) N=13; Enriched (1 pup) N=13; Enriched (3 pups) N=12. Data was analyzed by one-way ANOVA. *Statistically significant relative to baseline (all $p<0.001$).

5. DISCUSSION

The data from this project show that the animals exposed to the enriched environment displayed significantly longer maternal behavior sensitization latencies compared to animals exposed to the control environment. There was no significant effect of pup number. For the elevated "plus" maze (EPM) pre-test, there were no statistically significant differences between groups, but there was a trend towards less movement for the enriched group in basic, fine, and open arm movements compared to the control group. There were no significant differences between experimental groups for the plasma corticosterone levels, although the levels were significantly elevated in all experimental groups compared to the control indicating the maze post-test correctly acted as a stressor.

Animals in the enriched environment displayed higher levels of anxiety-like behaviors compared to animals in the control environment. These results are contrary to the expected results (Moncek et al., 2004), as it was predicted that the exposure to the enriched environment would have acted to mitigate stress, consequently reducing anxiety-like behaviors. First, animals in the enriched environment displayed significantly longer maternal behavior latencies. A possible reason for this result may be that the enrichment objects served as a distraction to the female rats, preventing the animals from being interested in interacting with the foster pups during maternal behavior testing. There were several cases in which the female remained inside the crawl ball for the entire duration of maternal behavior testing, without making any contact with the foster pups. For this reason, in a future study, it would be recommended to remove the enrichment objects from the cage before maternal sensitization testing is begun, so as to prevent the objects from serving as a direct immediate distraction to the females. In addition, enrichment objects that are interesting but do not allow the rat to "nest" in them, might provide a stimulating environment without completely occupying the animal's attention.

Another possible explanation for the longer sensitization latencies of the enriched animals is that the exposure to the novel enrichment objects could have initially increased the stress levels of the animals, leading to the observed opposite results. Because novel objects have been shown to act as a mild stressor in rats (Morimoto et al., 1992;

Chandramohan et al., 2007) it is possible that the exposure to the novel enrichment objects could have increased the stress levels of the animals to an extent where they would be less likely to explore other new objects, such as the pups. In this case, the females could have been less interested in interacting with the foster pups during maternal behavior testing, leading to longer sensitization latencies for the enriched group. In the future, perhaps serum cortisol levels could be monitored early in the enrichment period to determine whether stress levels are elevated by the enrichment procedure.

In addition to longer sensitization latencies, animals in the enriched group also displayed decreased movements in the open arms of the elevated plus maze during pre-testing. Movement in the open arms of the maze is indicative of exploratory behavior, and occurs more frequently when stress levels are reduced. It is possible that decreased movements in the open arms for the enriched group occurred due to the potential increased stress caused by the novel enrichment objects, causing the opposite of the expected result for the plus maze activity as it did for the sensitization latencies.

Ultimately, it is likely that the failure of the enriched animals to demonstrate reduced anxiety-like behaviors occurred because of inadequate initial exposure to the different experimental environments. In other environmental enrichment studies, in which environmental enrichment was shown to have positive stress-reducing effects, animals were initially exposed to the environments for 40 days (Kempermann et al., 1997) or as much as 2.5-3 months (Leggio et al., 2005). It is possible that one week of exposure to the enriched environment is not enough time for the enrichment to impose any stress-reducing effects. In a future study, it would be feasible to extend the period of time during which animals are exposed to the different environments.

With respect to pup number, experiments revealed that no major differences existed between the one pup and three pup groups. For the maternal sensitization latencies, there was no significant effect of pup number, and there were no significant differences in the corticosterone response to stress between the one and three pup groups. These results indicate that it is possible that the number of pups used in the maternal behavior sensitization paradigm could be reduced to one, resulting in a decreased number of animals necessary for testing.

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APPENDIX I: Maternal Behavior Testing Data Sheet

Environmental Enrichment Study
Maternal Behavior Testing Sheet

Date: ________ Test/Postpartum Day: ________________ Test Time: ________

	Pretest				Test					Post Test			
Rat #	**Nest**		**Pup Position**		**0 - - - - - - - - - -**			**30'**		**45'**		**60'**	
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
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					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								
					Con:	G:	B:						
					R1:	C:	W:						
					R2:	Notes:							
					R3:								

con = contact; R1 = retrieve first pup; G = group all pups in the nest; C = crouch over all 3 pups; B = in the ball; W = gnawing at wood