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Recent studies of early handling in inbred mice do not replicate results from previous work in rats. In addition, studies using extended periods of dam-offspring separation suffer from a lack of consistency in behavioral, physiological and neuroanatomical correlates. This study re-visited the maternal mediation hypothesis in an attempt to resolve some of these discrepancies. Three typical disruptions of mother-offspring relations were used: Early Handling (EH, daily 15 min separation), Maternal Separation (MS, daily 4 hr. separation from dam) and Maternal Peer Separation (MPS, daily 4 hr. separation from dam and littermates). These groups were compared to a weekly cage changed, Animal Facility Reared (AFR) group. MS & MPS dams displayed higher levels of nest attendance, quiescent nursing, activity in the nest and licking post-manipulation. In contrast to the levels of maternal care received, AFR offspring were found to be more emotional in the open field as compared to MPS offspring. Closer inspection of maternal behavior revealed substantial variability within treatment condition. Analysis of offspring behavior as a function of levels of maternal behavior revealed that pups that received high levels of quiescent nursing and activity but not licking were less “emotional”. Individual differences in pup licking behavior by dams was found to predict the variability in “emotional” behavior (in open field) for AFR and EH pups but not MS & MPS pups. Future studies employing these paradigms in inbred mice must examine individual differences in maternal care and its relationship to offspring behavioral development.

A RE-EVALUATION OF THE EFFECTS OF
MATERNAL CARE ON OFFSPRING
BEHAVIORAL DEVELOPMENT

by

Jeremy D. Bailoo

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Committee Chair

I dedicate this dissertation to my parents. I am thankful for your sacrifice, for it is because of you, that I have reached this far. I also dedicate this work to my wife. Without your steadfast encouragement and belief, this would never have been possible.

APPROVAL PAGE

This dissertation has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

Date of Acceptance by Committee

Date of Final Oral Examination

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CHAPTER I
A HISTORICAL REVIEW OF EARLY HANDLING AND MATERNAL
SEPARATION

Overview

Previous research in some stocks of the rat (e.g. Wistar, Sprague-Dawley) has demonstrated that a daily 15 minute separation (during PND 1-14) of the dam from the offspring (early handling (EH)) results in offspring that display a “blunted emotional” profile as adults. The characteristic pattern of dam-offspring behavioral responses as an immediate consequence of these brief bouts of maternal separation in these stocks of rats has been demonstrated to be mediated in part by the production of pup ultrasonic vocalizations post reunion with the mother. The pup’s ultrasonic vocalizations are subsequently followed by an up-regulation of patterns of maternal behavior (licking and arch-backed nursing) exhibited towards the pups. These maternal behaviors have, in turn, been associated with the remodeling of the Hypothalamic-Pituitary-Adrenal (HPA) Axis (increased numbers and density of specific neuromodulator receptors in the hypothalamus and hippocampus) in the pups. The increase in the number of receptors at the level of the hypothalamus and hippocampus, permit the rapid “turning off” of the HPA axis via negative feedback in response to a stressor; and thus the manifestation of a “blunted emotional” profile. These effects have been well documented, replicated and procedurally refined across time in rats and mice.

Interestingly, a conceptual and procedural (almost paradigmatic) shift in the investigation of this phenomenon in rodents occur around the 1970's, seemingly mediated by the work of Harry Harlow, Robert Hinde and John Bowlby who were investigating the effects of early maternal deprivation (long periods of maternal separation) in humans and non-human primate infants. This stimulated research in rats that used longer periods of mother offspring separation (between 3-6 hours) during PND 1-14. Such long separations (unlike EH) produced adult offspring who were “emotionally” highly reactive. The mechanisms mediating the differences that are observed as a consequence of these longer periods of separation are less well specified and the variability in behavior and physiology that is observed across studies appears to be a consequence of differences in the methodology used across laboratories.

The purpose of this dissertation therefore is to: 1) provide a historical overview of early handling and maternal separation in rats and mice; 2) evaluate the differences in procedures across labs and thus provide justification for the proposed study; 3) execute a study comparing short- and long-duration separations of mother and offspring in mice; 4) describe and evaluate our findings in light of previous research; and 5) propose a conceptual frame for the evaluation and construction of future research in this domain.

A Brief Historical Overview of Handling in the Rat

Between 1930 and 1950, at least fifty percent of reports in the Journal of Comparative and Physiological Psychology (formerly known as the Journal of Comparative Psychology and again divided into the “Journal of Comparative

Psychology” and the journal “Behavioral Neuroscience”) were performed using the Norway rat (Beach, 1950). Christie (1951) was the first to comment on the issue that experimental animals in these studies were generally described as “experimentally naïve”. He highlighted that the implicit notion in this description of experimental animals was that either an animal’s earlier experiences were irrelevant or that the contribution of any early experiential factors to the individual variability across rats was negligible.

Bernstein (1952), in an elaboration of Christie’s commentary, was first to formally describe the effects of an early experience manipulation that was commonly performed in laboratories, namely gentling or handling. He reported that laboratory reared albino rats that had been picked up and petted by the experimenter (i.e., gentling) for ten minutes daily subsequent to weaning (i.e., after postnatal day (PND) 21) until adulthood, performed better in a T-maze at PND 60 as compared to individuals who were handled from PND 50-60 (intermediate handling, (IH)) and to those who were not handled (NH) until testing. While previous to this study, it was “commonly understood” that this handling procedure subsequently resulted in adults who were easier to work with, this study was the first to empirically demonstrate that early experiences may contribute to the manifestation of different behavioral phenotypes.

Bernstein (1952) stressed that the age of initiation of handling as well as the amount of handling received across the two groups was confounded and thus the results should be interpreted with caution. Moreover, he argued that the superior performance of the handled animals may have been a consequence of an established relationship between the experimenter and the animal. Thus, when the animal was tested later, the mere

presence of the same experimenter reduced a secondary drive which served to reinforce the animal's behavior. This interpretation was derived from a mixture of learning and personality theory, and may be a consequence of the work of Miller and Dollard (1941) who assumed an analogous relationship between rats behaving in mazes and humans in everyday life situations, especially when interpreting the effects of early experience.

In 1953, and subsequently in 1954 and 1956, Weininger reported that albino rats handled post weaning (after PND 21) to adulthood had a greater body weight, greater skeletal length, and displayed a "less emotional" profile (increased ambulatory movement in an Open Field) as compared to non-handled controls. He described the handling manipulation as "enhancing the vitality of the albino rat" (Weininger, 1956), thereby "producing" an animal with a more resilient adult profile. Weininger's series of studies may have been motivated by Selye's (1950) stress theory and Hebb's (1949) theory which state that early experience affects brain development, because Weininger proposed that handling affected the maturation of the developing emotion/stress centers in the hypothalamus.

Concurrent with and parallel to this line of research, Seymour Levine was developing an animal model of early traumatic events (Levine, 2000). His first experiment (Levine, Chevalier & Korchin, 1956) subjected pre-weanling Sprague-Dawley rats to a mild shock (3 minutes in duration) during PND 1-20. One control group was a group of animals that was separated from the dam for 3 minutes a day but not shocked. An additional non-handled control group was used to evaluate whether disruption of the dam-offspring dyad contributed to any differences observed. Note that

Levine's handling manipulation occurred pre-weaning (early handling, (EH)), whereas previously, handling manipulations occurred post-weaning. In this study, Levine reported that only the non-handled controls as adults displayed poor learning on a conditioned avoidance task in a shuttle box (rapid avoidance learning).

Levine (1956) interpreted rapid avoidance learning as indicative of low "emotionality" and inferred that handling was a traumatic or stressful experience for the animal. The notion here was that early exposure to trauma (i.e., handling) raised the threshold for later responding to traumatic situations. Levine (2002) stated that this initial study was motivated by "the Freudian emphasis on the consequences of events during infancy for the development of psychological disorders". Thus, across the three laboratories (Bernstein, Weininger and Levine), three different interpretations were associated with the same treatment; i.e., handling resulted in the establishment of a relationship between the experimenter and the animal (Bernstein); "enhancing the vitality of the albino rat" thereby "producing" an animal with a more resilient adult profile (Weininger), and a traumatic or stressful experience for the animal which raised the threshold for later responding to traumatic situations (Levine).

The first empirical evidence that handling may have an effect on the Hypothalamic Pituitary Adrenal (HPA) axis came from a study by Levine (1957). In this study, male Sprague Dawley-Holtzman albino rat pups were either handled (n=27) or non-handled (n=29), respectively, during PND 1-20. On PND 70, twenty males from each group received a 20% glucose injection and were subsequently food and water deprived for 24 hours (a physiological stressor). A 20% increase in blood sugar levels in

the absence of water and food is a physiological stressor because it disrupts homeostatic balance and results in an upregulation of the HPA axis such that adrenocorticotrophic hormone (ACTH) secretions from the anterior pituitary and arginine vasopressin (AVP) or antidiuretic hormone (ADH) secretions from the posterior pituitary to the blood increase. Increased levels of circulating ADH serve to reinstate homeostatic balance by increasing water re-absorption and secretion of concentrated urine (antidiuresis). In this study, Levine found that non-handled subjects had a significantly greater adrenal weight as compared to handled subjects. This result was surprising because prior to 1957, adrenal hypertrophy in response to a stressor had been demonstrated to require longer than 24 hours between injection and adrenalectomy.

Levine and Otis (1958) were the first to have empirically challenged Weininger's finding that handling post-weaning resulted in offspring who were "more resilient" as adults when compared to non-handled controls. In this study, 5 groups of Sprague-Dawley rats were examined: 1) early-stroking during PND 1-21 (subjects were stroked for five minutes daily); 2) early-handled during PND 1-21 (subjects were removed from the nest, weighed, and returned to the home-cage); 3) stroked during PND 21-42 (subjects were stroked for ten minutes daily); 4) handled during PND 21-42 (subjects were removed from the nest, weighed, and returned to the home-cage); 5) a non-handled control group. This study failed to replicate the finding that early stroking or handling post-weaning produces offspring which were "more resilient" as compared to non-handled controls. Instead, only early stroking or handling pre-weaning was shown to

promote “resiliency”. Resiliency was demonstrated by a greater body weight and a greater likelihood to survive following deprivation.

In the six decades following these initial studies, the effect of handling on the rearing environment of offspring as well as the behavior, physiology and neuroanatomy of offspring development have been well documented. The subsequent sections summarize these findings and attempt to link these bodies of research.

The effects of Early Handling in Rats

- ***The Theory behind the Logic***

As noted above, the term “handling” has been used to describe a diverse range of treatments. One common factor in all of these treatments was the direct contact between experimenter and the animal. However, the notion that this early handling effect depended on contact between experimenter and the animal was derived from *a priori* assumptions and theories which may have biased the methodology used in the application of the handling treatment as well as the interpretation of its effects.

Levine (2000) states:

Science, like most other endeavors, is influenced by the culture of the time. When these studies were conducted, it was believed that some compound existed that was involved in regulating the expression of emotions. One of the logical candidates for this compound was the adrenocortical hormones. The most influential and pervasive thinking concerning stress physiology at the time was Selye’s formulation of the General Adaptation Syndrome (GAS), which gave a central role to the adrenal hormones as one of the predominant responses to stress.

It is therefore worthwhile to briefly describe the historical roots of Selye's theory. The term "stress" (in behaving organisms) was coined by Hans Selye. He was born in Vienna in 1907 and during his second year of medical school, he began working on his theory of how stress affects the way that people adapt to and cope with injury and disease. He observed similar symptoms in individuals in the early stages of infectious diseases, irrespective of the disease. He termed this characteristic pattern of responding as the General Adaptation Syndrome (Selye, 1946). Selye (1956) explained that he:

called this syndrome *general* because it is produced only by agents which have a general effect upon large portions of the body. I called it *adaptive*, because it stimulates defense. I called it a *syndrome* because its individual manifestations are coordinated and are even partly dependent upon each other.

Thus, the initial work by Levine and colleagues on the effects of early handling on the developing offspring was an attempt to create an animal model of early traumatic events. In particular, for Levine, perhaps the most important motivating factor for creating an animal model of early life stress was the central role given to adrenocortical hormones in the formulation of the GAS (Levine, 2000). As described previously, confirmation that early handling affects the HPA axis (Levine, 1957) led to a series of experiments by Levine and others into the mechanism via which handling leads to the manifestation of a "resilient/less emotional" behavioral profile.

Hunt and Otis (1955, 1963) were the first to demonstrate that placement of albino rat pups in a cage post separation for a few minutes produced the same effects of several minutes of holding and stroking the pup in the experimenter's hand. In this experiment, thirty nine Sprague-Dawley Holzman albino rats were either handled (n = 19) twice daily

during PND 7-21 or non-handled (n = 20) until PND 21. Their handling procedure involved picking the pup up from the home cage and placing it in cage with sawdust litter from the home cage (note: all handling procedures described previously involved holding the pup in the experimenter's hand and stroking the animal). On PND 339, animals were food and water deprived for 24 hours. They were then administered a "timidity" test where "the motivated subject was to emerge from the standard wire starting cage and retrieve one or more pellets of food placed 12, 20, and 30, inches out on a runway extending from the front of the cage" (Hunt & Otis, 1955). Individuals who emerged completely from the starting cage and obtained the pellet of food were classified as "less emotional." The results demonstrated that handled subjects were "less emotional" than non-handled subjects.

This study, as well as the study by Levine and Otis (1958) (described in the previous section), are noteworthy because they both used a more inductive approach to explore the mechanism behind the effects of handling. That is, unlike the other studies on early handling, the experiments by Hunt and Otis (1955) and Levine and Otis (1958) were not derived entirely from theory but were designed to explore the treatment itself. In fact, these two studies seemed to reorient subsequent investigations into the early handling phenomenon toward more focus on the mechanisms involved.

- ***The Critical Period***

Schaefer (1963), in a follow-up study to Levine and Otis (1958), sought to define the age at which handling has the greatest effect. Schaefer (1963) assigned two litters of

Sprague-Dawley rats to each of the following five treatment groups: 1) Pups handled daily during PND 1-21; 2) Pups handled daily during PND 1-7; 3) Pups handled daily during PND 7-14; 4) Pups handled daily during PND 14-21; and 5) a non-handled control group. The handling procedure consisted of removing pups individually from the nest cage, holding each pup in the hand for two minutes, and then placing the pup on a tray of shavings for two minutes. The litter was then returned in its entirety to the natal nest with the dam. Pups were tested on “emotionality” in the open-field at PND 55. They found that only males that were handled in the first week, males that were handled for all three weeks, and females that were handled for all three weeks demonstrated decreased emotionality (reduced crouching and defecation in an open field subsequent to the presentation of a loud click). Additional confirmation of the maximal effect of early handling during the first week of life was provided by Levine and Lewis (1959) using Sprague-Dawley rats and by Denenberg and Karas (1960) using Wistar rats.

In summary, it was proposed that a critical period exists in rats for which the maximal effects of early handling are manifest. In other words, during the first week of life (i.e. PND 1-7), removal of the pups for 5-10 minutes daily “produces” adults who display a blunted “emotional” profile. In the years following the ascription of this critical period, many hypotheses were tested in an attempt to explain the mechanism behind these effects. These hypotheses are reviewed below.

- *Perceptual Capacities or Emotionality?*

Hebb (1949) proposed that the reaction of an adult animal to its environment may be a consequence of its early visual experience. Many studies have demonstrated the necessary role of “experience” in visual perception. For example, Gibson et al. (1959) found that Albino rats reared from birth to PND 90 in the dark weighed less and displayed poor consummatory behavior in a novel environment. Walk (1960) found that Lister hooded rats reared from birth to PND 90 in the dark were not significantly different from light-reared subjects in terms of their tendency to approach a novel arm in a T-maze after repeated testing. However, dark-reared subjects were classified as “more emotional” due to their tendency to not enter an arm completely. Tees (1969) found that Long Evans rats reared in the dark from birth until PND 65 were less ambulatory and defecated more when tested in an open field on PND 90. Dark reared animals were described as “more emotional” due to their increased defecation.

Researchers investigating the mechanisms of the early handling phenomenon were quick to note the similarities in the responses to dependent measures between the studies used in early visual perception and those used in handling. Thus, another popular theme at the time involved the question of whether better visual ability was related to the lower emotionality observed in handled rats. That is, does a more adequate ability to discriminate in a testing environment lead to reduced emotionality?

Denenberg and Mutton (1962) were the first to report on this question. Early handled (pre-weaning) and non-handled Wistar rats were reared in enriched, neutral or restricted housing environments post-weaning. Subjects were trained on the Hebb-

Williams maze on PND 51-65 and tested on the maze beginning at PND 66. As no differences were observed between the subjects reared in neutral and restricted environments, the data for these subjects were collapsed across handling conditions. The authors found that early handled subjects were not significantly different from their non-handled counterparts in terms of their problem solving behavior. Schaefer (1963) subsequently replicated this experiment using Sprague-Dawley rats with the same result. Thus, visual discrimination ability did not appear to account for the differences observed in the open field testing situation between early handled and non-handled rats.

- *The effect of Temperature*

Schaefer et al. (1962) was the first study to report on the effects of temperature on later “emotionality” in the absence of handling. Thirteen litters of Sprague-Dawley rats (118 pups) were assigned by litter to four treatment conditions: 1) A group that was handled for 2 minutes daily during PND 1-7 (n=30 pups); 2) a non-handled control group (n=31); 3) a cold exposed group that was exposed to 7-10°C temperature (via placement into a working refrigerator; n=31) and; 4) a cold control group (placed in a non-working refrigerator and kept at 23°C, n=26). The number of litters ascribed to each group was not reported. Levine, Alpert and Lewis (1958) had previously demonstrated the earliest age at which significant depletion of adrenal ascorbic acid by cold stress is observed on PND 12. Thus, on PND 12, half of the subjects from each of the four groups were randomly assigned to be cold stressed. Pups were removed from their cages, placed in small individual stainless-steel compartments, and subjected to a cold stress of 5°C for 90

minutes. Subjects were then killed via cervical dislocation, and their adrenals were removed and assayed for levels of ascorbic acid. Schaefer et al. (1962) stated that “depletion of adrenal ascorbic acid in response to cold stress was selected to evaluate the effectiveness of treatments because it yields clear-cut differences between handled and non-handled animals at an early age, and because it permitted us to replicate some of Levine's excellent work.” The authors found that the handled and the cold exposed group showed a significant reduction of adrenal ascorbic acid in response to cold stress as compared to the non-handled and cold control groups. This suggested that an essential aspect of handling is the associated decrease in pup temperature.

Subsequently, Schaefer (1963) reported on whether the effects of handling would persist in the absence of a temperature change. Twelve litters of Wistar Albino rats were assigned to three treatment groups: 1) A non-handled control group; 2) A warm handled group removed from the natal nest on PND 2-5 and placed for eight minutes in an incubator at 34-36°C and; 3) A cold handled group removed from the natal nest on PND 2-5 and placed for eight minutes in an incubator at room temperature, 22°C. The warm-handled temperature of 34-36°C was empirically determined to be representative of the natal nest temperature while the temperature of 22°C was chosen to be representative of the ambient temperature during many handling studies. On PND 13, half of the subjects from each of the three groups were randomly assigned to be cold stressed. Pups were removed from their cages, placed in small individual stainless-steel compartments, and subjected to a cold stress of 5°C for 90 minutes. Subjects were then killed via cervical dislocation, and their adrenals were removed and assayed for levels of ascorbic acid. The

results indicated that the group that had been handled at room temperature had a depletion of ascorbic acid which was five times greater than the non-handled and warm handled groups. This provided further support that the effects of handling depended on a corresponding decrease in pup temperature.

In contrast to the Schaefer studies, Hutchings (1963) was interested in developing “a sensitive, quantitative behavioral measure for evaluating the effects of various early treatments related to the handling procedure.” This experiment was noteworthy because, like Schaefer (1962, 1963), emphasis was placed on exploration of the treatment itself. Twelve litters of Sprague-Dawley albino rats were assigned equally (i.e., 6 litters per group) to either a non-handled control group or an early treatment group. Pups in the early treatment group were removed from the dam daily on PND 1-7 and placed individually in a metal can standing in a water bath at 8-12°C for five minutes. Hutchings (1963) acknowledged that his experimental manipulation emphasized a decrease in temperature rather than handling itself. This, however, does not negate the fact that this study confounds temperature and handling, and should therefore be interpreted with caution.

In this study, offspring were weaned at PND 21 and only the male subjects were tested as adults. On PND 85, subjects were maintained on a daily 23 hour water deprivation schedule until PND 90. The behavioral measure of emotionality used in this experiment was a modification of the paradigm developed by Mowrer and Aiken (1954). In this method, a conditioned stimulus previously paired with shock is made contingent on the lever pressing response originally established for food or water reward. Even

though the water reward is continued, introduction of the conditioned stimulus has been demonstrated to suppress lever pressing (Mowrer & Aiken, 1954). The duration of lever pressing suppression is purported to be indicative of the “intensity of the emotional disturbance produced by introduction of the conditioned stimulus” (Hutchings, 1963).

Thus, on PND 90, subjects were trained on a lever pressing task for continuous water reinforcement (CWR) in daily 45 minute free operant sessions. Training continued until all animals exceeded a performance criterion of ten or more lever presses per minute for three consecutive minutes. This was followed by two daily 30 minute CWR lever pressing runs for response stabilization. Thereafter, daily 30-minute lever pressing runs (LPR) for water were continued. In order to establish an emotional response, an emotional conditioning (EC) trial, consisting of the presentation of one pairing of light and shock, was administered once on three different days in place of the daily LPR. One or more daily LPR trials were administered after each day of emotional conditioning, and before the next emotional conditioning trial. Daily LPR was continued until the latency to begin lever pressing did not exceed ten minutes. The sequence of trials across days for all animals was as follows: EC, LPR, EC, LPR, LPR, LPR, EC, LPR, LPR.

After this procedure was completed, subjects were placed in Skinner boxes under normal lever pressing conditions for one or more 60 minute runs. When subjects emitted a high stable rate of lever pressing, the experiment was started. In addition to water reward, three seconds of the conditioned stimulus light (without shock) was presented. This resulted in a complete suppression of lever pressing, as well as crouching and immobility presumably due to its previous pairing with shock. With continued

unreinforced occurrences of the conditioned stimulus across the trial, the emotional response was extinguished and a normal rate of lever pressing was resumed. Emotionality was operationally defined from the duration of lever pressing suppression.

The results indicated that the early treated group took a significantly longer time to acquire lever pressing to performance criteria, had significantly longer latencies to begin lever pressing on days subsequent to the conditioned-unconditioned stimulus pairing, and had significantly longer durations of lever pressing suppression following the introduction of the conditioned stimulus during testing for “emotionality” as compared to the non-handled controls. This was the first study to provide evidence that animals handled pre-weaning were *more* emotional than non-handled controls. However, as handling and temperature were confounded in this study, the results should be interpreted with caution.

This paradoxical finding led Schaefer (1963) to hypothesize that the early treated animals in the study by Hutchings (1963) had been subjected to a more dramatic change in temperature, thus producing a reversal of the handling effects. He replicated Hutchings (1963) experiment and added an additional treatment group. In the additional group, subjects were placed individually on a tray of wood shavings for five minutes daily during PND 1-7. Thus, Schaefer’s study permitted dissociation between the effects of handling and temperature. Like Hutchings (1963), Schaefer found that cold treated animals were more emotional than non-handled controls when tested on the extinction of conditioned fear. Furthermore, as he predicted, the group that was handled on a tray of wood shavings at room temperature was less emotional than the non-handled controls.

The results of this experiment led Schaefer (1963) to hypothesize that a curvilinear relationship exists between emotionality and temperature change in response to handling during the critical period (PND 1-7). Hutchings (1967) expanded on this notion of a curvilinear relationship between handling and temperature change. He randomly assigned by litter thirty three Sprague-Dawley litters (with a maximum litter size of ten and a minimum of eight) to one of seven treatment conditions: 1) a 10 minute warm handled group maintained between 34.5-35.5°C (WARM) (n = 16 pups); 2) a 10 minute cold handled group maintained between 27.5-29°C with a net loss in body temperature of 3-4°C (SLO 10) (n = 19 pups); 3) a 3 minute cold handled group maintained between 27.5-29°C with a net loss in body temperature of 3-4°C (SLO 3) (n = 19 pups); 4) a 3 minute cold handled group between 22-23°C with a net loss in body temperature of 3-4°C (MOD 3) (n = 23 pups); 5) a group that was cold handled for 3 minutes at 22-23°C and then warm handled at 27.5-29°C for 7 minutes (MOD 3-7) (n = 16 pups); 6) a group that was cold handled for 1 minute at 5-6°C with a net loss in body temperature of 3-4°C and then warm handled for 9 minutes at 27.5-29°C (RAP 1-9) (n = 24 pups) and; 7) a control group (AFR) which received only the standard animal husbandry as the other groups (n = 29 pups). Changes in body temperature reflected the 5 day average across treatment and were measured on the lateral abdominal surface using a rapid registering surface probe and telethermometer. It was unspecified how litters were divided across treatment conditions and it should be noted that the number of pups reported for each condition were comprised of only male offspring.

Male subjects were tested on “emotionality” in the open-field at PND 55-70. The dependent measure was a comparison of the total time spent crouching before and after the presentation of a loud auditory stimulus (duration unspecified). Data were analyzed using the Mann-Whitney *U* test. The results indicated that only the RAP 1-9 group crouched significantly less than the AFR group. Also, groups SLO 3, MOD 3 and MOD 3-7 crouched for a significantly shorter duration than the WARM group. Similarly, compared to the SLO 10 group (which was not significantly different from the WARM group), SLO 3, MOD 3, MOD 3-7 and RAP 1-9 all crouched significantly less. The RAP 1-9 group crouched significantly less than the WARM, SLO3 and MOD 3-7 groups and no significant differences were observed between the groups MOD 3, MOD 3-7 and SLO 3.

In concordance with the findings reported by Hutchings (1965) and Schaefer (1963), no significant differences were observed between the WARM group and non-treated controls. Thus, handling in the first week of life seems ineffective in affecting “emotionality” as adults, unless a corresponding decrement in temperature occurs. The reduced emotionality observed in MOD 3 in comparison to the WARM group replicates the findings of Hutchings (1963) and is comparable to the findings of other researchers (e.g., Denenberg et al., 1962).

The group SLO 10 was not significantly different from the AFR control group, thus replicating the finding by Hutchings (1965). The increased crouching score of the SLO 3 group that was significantly lower than the SLO 10 group (i.e., a reversal of the effect as they were both exposed to the same temperature, albeit for different durations)

lends support for a curvilinear relationship between the duration of cold exposure and subsequent “emotionality”. This curvilinear relationship is also supported by the lack of a difference in crouching between the MOD 3 to MOD 3-7. Lastly, comparison of the SLO 10 to the MOD 3 and MOD 3-7 groups (which underwent a 3-4°C temperature loss continuously over 10 minutes vs. 3 minutes) demonstrated that the SLO 10 group spent significantly more time crouching than either MOD groups.

Hutchings (1967) stated that "as maintenance at a level of hypothermia 3 to 4°C below normal does not appear to contribute to later effects, it is tentatively assumed here that the dramatic reduction in emotionality obtained for Group RAP 1-9 was produced by the initial 1 minute exposure to 5 to 6°C and the resulting 3 to 4°C temperature loss”. This interpretation, coupled with his earlier finding (Hutchings, 1963) that a 5 minute exposure to 8-12°C gave rise to pups that were behaviorally more “emotional” as adults, fits with the hypothesized curvilinear relationship between the effects of handling and temperature change (where a brief exposure to low temperatures reduces “emotionality” and prolonged exposure to low temperatures increases “emotionality”).

Several aspects of this study should be noted. The first is that the control group is different from all of the previously reported studies. This control group has subsequently been referred to in the literature as an Animal Facility Reared (AFR) control group. All of the previously described studies used a non-handled (NH) control group, which receives no animal husbandry until weaning. Thus, any differences that emerge from comparisons to the AFR control group in this study are not directly comparable to those espoused in other studies which use a non-handled control group. The implications of the use of a

different control group will be discussed in a later section. Second, in order to control for the additional effects of experimenter manipulation between groups that received two temperature treatments (i.e., MOD 3-7, RAP 1-9), each pup in the single temperature treatment (i.e., WARM and SLO 10) was individually lifted out of the holding container and quickly replaced after 3 minutes. Third, the dam was removed from the cage for the entire duration of each treatment and was returned following replacement of the litter. Also, a plastic bag filled with water heated to 34-35°C was placed in each natal nest when the litter was absent. Thus, litters were returned to a warm nest and the dam to a warm litter.

It is also worth noting that significant differences in responding to these early treatments were observed even though pups in the chilled groups were “re-warmed” before returning the pups to the dam. It is unspecified whether the mother may have been responding to stimulus characteristics induced by cold exposure other than the lowered temperature of the litter, such as ultrasonic vocalizations. Furthermore, at this junction in history, it had yet to be demonstrated that altered maternal behavior as a consequence of the handling treatment was responsible for the change in litter “emotionality”.

In a second experiment, Hutchings (1967) investigated whether the curvilinear relationship described previously may be a function of rate of temperature loss. Thirty two Sprague-Dawley litters (with a maximum litter size of ten and a minimum of eight) were randomly assigned by litter to one of seven treatment conditions (4-5 litters per treatment): 1) a 3 minute cold handled group maintained between 22-23°C (MOD 3) with a net loss in body temperature of 3-4°C; 2) a 5 minute cold handled group maintained

between 22-23°C (MOD 5) with a net loss of 4-5°C; 3) a 10 minute cold handled group maintained between 22-23°C (MOD 10) with a net loss in body temperature of 5-6°C; 4) a 1 minute cold handled group maintained between 5-6°C (RAP 1) with a net loss in body temperature of 3-4°C; 5) a 3 minute cold handled group maintained between 5-6°C (RAP 3) with a net loss in body temperature of 5-6°C; 6) a 5 minute cold handled group maintained between 5-6°C (RAP 5) with a net loss in body temperature of 9-10°C; 7) an AFR control group which received the same standard animal husbandry as the other groups. Again, changes in body temperature reflect the 5 day average across treatment and were measured on the lateral abdominal surface using a rapid registering surface probe and telethermometer. Unlike experiment 1, both males and female were tested as adults.

Subjects were tested on “emotionality” in the open-field at PND 25-28. Hutchings (1967) stated that “preliminary study indicates that the effects can be measured soon after weaning.” The dependent measure was a comparison of the total time spent crouching before and after the presentation of a loud click (duration unspecified). An over-all curvilinear relationship between duration of cold exposure and subsequent crouching in the open field was observed when the crouching scores of the non-treated and experimental groups exposed to 22-23°C and 5-6°C, respectively, were compared. Visual analysis of the trends in crouching scores revealed that the initial effect of exposure to cold was a reduction in crouching time. Exposure beyond the treatment duration which produced the maximum reduction in crouching (i.e., 5 minutes for groups exposed to 22-

23°C and 3 minutes for groups exposed to 5-6°C) reversed the direction of the effect, returning scores to the non-treated level.

Additionally, planned orthogonal contrasts indicated that individuals in the RAP 1 and RAP 3 groups crouched significantly less than the MOD 3 and MOD 5 groups. Thus, it appeared that brief exposure to 5-6°C is more effective in reducing emotionality than longer exposure to 22-23°C. That is, groups exposed for 5 minutes to a temperature of 22-23°C had the greatest reduction in crouching while the group exposed to the same temperature for 10 minutes crouched similarly to the non-treated group. Similarly, groups exposed for 3 minutes to a temperature of 5-6°C had the greatest reduction in crouching while the group exposed to the same temperature for 5 minutes crouched similarly to the non-treated group. Thus, the more rapidly body temperature is lost, the more rapidly curvilinearity (in regards to “emotional” behavior) is obtained.

In summary, by 1967, the research evidence supported the notion that the temperature of early handled pups post separation is a major factor in the manifestation of a “less emotional” adult behavioral profile. This relationship between temperature loss and later adult “emotionality” seems to be best described as curvilinear. Furthermore, the more rapid the temperature loss, the more likely curvilinearity is obtained.

- ***Separation of Pups from the Dam***

As noted in the previous section, a decrease in pup temperature appears to be an important factor mediating the effects of Early Handling. One question that remained unclear was *how* a decrease in pup temperature results in decreased emotionality of

offspring as adults. One hypothesis, which initially received little support but subsequently became recognized as an important mediator of the effects of handling, was that handling of the pups may affect the way in which the dam interacts with her young post reunion.

Schaefer (1959) was the first to examine the effects of variation in length of disruption of the dam offspring dyad. Schaefer (1968) stated that the “separation of the mother and pups was a factor confounded in all handling studies at the time of the present study”. Recall, Levine et al. (1956) had demonstrated that animals that were shocked during infancy displayed a similar behavioral profile to that of handled animals. Levine et al. (1956) interpreted that the handling operation itself was a traumatic experience for the developing rat pup. Schaefer in (1968) stated that in his 1959 study “it was hypothesized, therefore, that separation from the mother may be a crucial factor in handling, providing a traumatic experience similar to electric shock”.

Schaefer (1959) compared 5 treatment groups (three litters per group) of a genetically inbred strain of Sprague-Dawley rats: 1) a non-handled control group; 2) a group handled from PND 1-21; 3) a group where the dam was removed from the nest for ten minutes each afternoon between PND 1-21; 4) a group where the dam was removed from the nest for 6 hours daily during PND 1-21 and; 5) a group where the pups were whelped and housed in modified cages which necessitated that the dam leave the natal nest cage to obtain food and water. Pups were tested on “emotionality” (i.e., crouching and defecation in an open field subsequent to the presentation of a loud click) in the open-field at PND 55. The only significant difference observed was between males in the

3 week handled group compared to the non-handled controls. The results of this experiment should be interpreted with caution as the sample sizes across all five groups were largely uneven (e.g., 20 in the NH Female groups vs. 4 in the Handled for 3 weeks Female group). Schaefer (1959) suggested that the results of this study indicate that the separation of the dam from offspring is not a pertinent aspect of the handling manipulation.

Du Perez (1964) also provided some evidence that the effects of handling may not be maternally mediated. His study investigated three hypotheses: 1) whether the effects of handling on adult “emotional behavior” were persistent; 2) whether the size of the housing cage post weaning had an effect on the reported behavioral changes consequential on handling and; 3) whether the daily removal of the dam had an effect on the pups. It is important to describe the three questions investigated in this study as well as the methodology used, as this may have affected the interpretation of the results.

One hundred and twenty-six Wistar pups from an undisclosed number of litters were assigned to five treatment groups: 1) early handled and housed in a small cage (n=30); 2) early handled and housed in a large cage (n=18); 3) undisturbed and housed in a small cage (n=30); 4) undisturbed and housed in a large cage (n=18) and; 5) dam removed daily for 15 minutes, untouched by hand and housed in small cages (n=30). Handling occurred from PND 1-25 (recall that this was around the time the critical period hypothesis was still under investigation). The authors mentioned in passing that the dams were gentled during gestation but did not discuss whether this may have had an effect on their subjects. Subjects were weaned on PND 25, and were separated into three groups

for testing at different time points (PND 50, 100 and 220) in adulthood on avoidance learning and learning for water reward on an elevated T-Maze. These group sizes were equivalent across the six treatment conditions.

Avoidance learning was assessed in a glass walled box divided into two equal compartments by a perspex wall with an aperture at a height of 5 inches. Each subject was given 3 preliminary runs of 3 minutes (across 3 days) in the avoidance training apparatus. During these runs, the perspex wall was removed and free exploration of the apparatus was permitted. No shock was presented during these runs, and counts of rearing, crossing between compartments, latency to first crossing in each 3 minute run, grooming, and defecation were recorded. Avoidance training was then commenced at a rate of two trials daily (with a 90 minute interval between trials) until a criterion response of two successive avoidances of shock by escape to the adjacent compartment was observed. Each avoidance training trial proceeded as follows. The subject was placed in the starting compartment of the box (the side with the electrified floor) and a buzzer was sounded for 5 seconds. The floor was electrified 5 seconds after the buzzer stopped. The subject was assisted through the aperture if it failed to escape after 50 seconds. For each subject, both the number of trials required to establish the avoidance response and the latency to response were recorded. Extinction trials followed a schedule identical to avoidance training. These trials commenced on the day subsequent to learning the avoidance response and continued until a criterion response of two successive “no-responses” of 30 second duration each was observed. For each subject, the number of

trials required to extinguish the avoidance response and the latency to response were recorded.

Subjects then learnt the elevated T-Maze (1 trial per day for 5 days) for water reward subsequent to 21 hours of water deprivation. Subjects remained in the apparatus until the reward of 10 seconds of water consumption was achieved. The time taken to reach the goal and to consume the water was recorded for each subject.

DuPerez (1964) found that the rate of growth was not affected by any of the treatment conditions. Handled animals were characterized as “less emotional”, as they were found to rear more, groom less, require less time to learn the avoidance task, and, at least when tested on PND 220, required less time to extinguish their avoidance response as compared to undisturbed animals. No measure of activity was affected by the cage size or removal of the dam. This result suggests that the removal of the dam during the process of handling does not mediate the effects of handling. However, in this study as in the one by Schaefer (1959), it is unclear whether the behavior of the dam post reunion with the pups may have mediated the effects of early handling.

Young (1965) was the first to suggest that stimulus changes in the pups as a consequence of early handling may affect the maternal behavior of the dam post reunion. He found that when post-parturient dams were given a choice between hypothermic pups and controls, the dams preferred the control pups (as indexed by retrieval). It is worth noting that it is unclear, other than in an anthropocentric sense, whether pup retrieval is a necessary variable in the umbrella of behaviors encapsulating “maternal responsiveness in the rat.” That is, it has yet to be empirically demonstrated that pup retrieval to the natal

nest contributes to the differential reproductive success of individuals that comprise the various species or genera in the family *Muridea*. That is, in the native habitat, how often does pup-retrieval happen or is it a phenomenon created in the laboratory?

Thoman and Levine (1969) were the first to directly implicate the dam's behavior in consequences associated with early handling. In their study, seven Sprague-Dawley females were mated with Long Evans male rats, thus generating seven litters. These 7 litters were assigned to 6 treatment groups: 1) a handled group (CC) where the pups were kept at room temperature during separation; 2) a warm handled group (WW) where the pups were kept at $37^{\circ} \pm 0.5^{\circ}\text{C}$ during separation; 3) a handled group (WC) where the pups were kept warm for the first 5 minutes of separation at $37^{\circ} \pm 0.5^{\circ}\text{C}$ and then allowed to cool to room temperature for the remaining 5 minutes; 4) a handled group (CW) where the pups were kept cool for the first five minutes and then kept warm at $37^{\circ} \pm 0.5^{\circ}\text{C}$ for the remaining 5 minutes; 5) a group where the dam was removed from the cage for ten minutes and; 6) a non-handled control group. All handling procedures occurred daily during PND 2-7.

Subjects were weaned on PND 21 and separated according to sex and experimental treatment. Offspring were tested for four consecutive days in an open field on PND 90. Subjects were tested in a counterbalanced order for treatment condition and sex. Following the last open field day of testing, subjects were placed in a holding cage for 12 minutes and then rapidly decapitated. Trunk blood was collected and assayed for plasma corticosterone concentration. No significant differences were observed across group in terms of the number of grids crossed in an open field or the presence of

defecation. These behavioral measures agree with results found by Schaefer (1963). However, a different picture emerged in regards to subject's adrenal corticosterone response to stress. With the exception of the CC females, each of the nine other groups was found to be significantly different from their like-sex control group. Regardless of temperature and handling condition, if the dam was separated from the pups, a significant difference in mean adrenal corticosterone response to stress was observed in comparison to non-handled controls for both males and females. This finding directly implicates the dam in the effects observed in response to the early handling treatment.

Based on the results of the study by Thoman and Levine (1969), Levine (2002) wrongly concluded that temperature is not a necessary variable in the effects of handling. Any change in behavior necessitates a change in physiology and vice versa. Thoman and Levine (1969) found a difference in physiology across groups, but no corresponding differences in behavior. Over 30 years later, Levine (2002) argued that the behavioral test used in the 1969 experiment was not sensitive enough to detect the corresponding changes that were detected by the changes in physiology across groups. However, this is an empirical issue that can only be resolved by additional research.

In 1969, Hess et al. (1969) tested the hypothesis that early handling of pups affected adrenocortical activity in pups as adults and was not a consequence of the absence of the dam. They assigned Wistar rats to four groups: 1) early handled on PND 1-5 at 22°C; 2) early handled on PND 1-20 at 22°C; 3) early handled on PND 1-5 at 35.5°C and 4) a non-handled control group. Subjects were weaned between PND 21-23 and housed four per cage (based on sex and treatment condition). Subjects were tested on

PND 35-37 in an open field for three minutes. One subject from each cage was not tested and was used instead to estimate baseline levels of corticosterone. The remaining three subjects in each cage were separated and placed individually in open fields for testing. Following testing, the three subjects were returned to their cages for 12, 27 or 57 minutes. Blood was collected at these time points via decapitation and assayed for plasma corticosterone (as in the baseline subject).

Similarly to Thoman and Levine (1969) and Schaefer (1963), no significant differences in behavior (locomotor activity in an open field) were observed across all treatment groups. Also, like Thoman and Levine (1969), a different picture emerged in terms of the adrenocortical response. Across all handled treatments, a significant reduction in the plasma corticosterone response across time was observed. Hess et al. (1969) noted the lack of concordance between their behavioral and physiological measure and stated that “the measures used in the open field did not discriminate across the groups.” Moreover, they acknowledged that the “open field data are difficult to interpret”.

Lee and Williams (1974) were the first to report on differences in maternal behavior as a consequence of early handling. They found that when a dam was reunited with the pups, there was an immediate approach response, followed by sustained elevated levels of maternal care. These behaviors included licking/grooming (LG) and arch backed nursing (ABN). Observation of the post reunion shift in behavior has subsequently been independently validated (e.g., Liu et al., 1997). It is currently unknown whether this observed change in maternal behavior is a function of changes in maternal physiology,

changes in the strategies of the pups to elicit maternal care, or a combination of both of these factors.

Finally, there is some evidence which suggests that the long term effects of handling are at least partially mediated by the changes in tactile stimulation provided to the pups from the dam as a consequence of handling (Levine, 1975; Smotherman, 1983). For example, Denenberg et al. (1968) have demonstrated that increased intensities of maternal care can reduce stress reactivity (as measured by high levels of activity in open field) in subjects as adults. D'Amato et al. (1998) have found that dams treated with benzodiazepine do not display a compensatory increase in maternal behavior upon reunion with the pups, and these pups, as adults, do not display the classic resilient "emotional" profile associated with handling.

In summary, the research suggests that the stimulus properties of the pup upon return to the mother may elicit differences in maternal care that can affect the manifestation of a less emotional behavioral profile in adult animals that are handled as pups. This relationship is contingent on the presence of the dam in the natal nest and has been linked to a compensatory increase in licking/grooming and arch backed nursing.

- ***The effects on the HPA Axis***

Thus far, I have outlined research that demonstrates that a critical period exists during which the effect of early handling on adult emotionality is maximized in rats. Also, research demonstrates that temperature of the pups (stimulus properties) post reunion, may affect the way in which the dam interacts with the young. There is some

evidence indicating that the mother may mediate the changes associated with early handling. Furthermore, some evidence indicates that the difference in stimulus properties of the handled versus the non-handled pup may contribute to the development of the pup's Hypothalamic-Pituitary-Adrenal (HPA) axis, also commonly known as the stress axis. It is therefore important at this juncture to describe the HPA axis and outline the evidence which compelled researchers to investigate the effects of early handling on this axis.

Sometimes termed the “flight or fight response system”, the HPA axis exhibits high responsiveness to any situation that is potentially dangerous to the individual. In many instances, a situation is treated by the HPA responsiveness as dangerous although there is little likelihood of physical damage to the individual. These situations often are labeled as “stressful” because they elicit activity in the individual's HPA axis similar to that elicited by a potentially physically harmful situation. The specifics of the situation have been called a “stressor”.

During a stressful event, the hypothalamus releases corticotrophin releasing factor (CRF) from the paraventricular nucleus. Vasopressin, as well as co-secretagens like oxytocin and angiotension II from neurons located in the supraoptic and paraventricular nucleus of the hypothalamus, are also secreted into the blood system of the posterior pituitary. CRF release leads (via the hypothalamic-hypophyseal portal system) to an increase in the release of adrenocorticotropin (ACTH) from the anterior pituitary, which travels through the blood to the adrenal glands and results in the secretion of mineralocorticoids (involved with the retention of sodium) and glucocorticoids (involved

with the uptake and metabolism of glucose) from the adrenal cortex. The adrenal cortex is also a secondary site of androgen synthesis.

Glucocorticoids are highly catabolic and stimulate lipolysis (which increases the levels of free fatty acids), glycogenolysis (which increases blood glucose) and protein catabolism (which increases the levels of amino acids available for gluconeogenesis). Thus, one immediate effect of high levels of circulating glucocorticoids is to increase the availability of substrates essential for cells to do work.

Another immediate effect of high levels of circulating glucocorticoids is to suppress immunological responses, which prevents inflammation when mobility may be essential. Continued exposure to high levels of circulating glucocorticoids may be detrimental to the organism (c.f., Selye, 1950, 1956). These effects include but are not limited to decreased insulin sensitivity, muscle atrophy, hypertension and immune-suppression (Baxter & Tyler, 1987). Thus, upon termination of a “stressor”, it appears that it may be beneficial for an organism to “turn off” the HPA-axis. Rapid regulation of the HPA response to a stressor may signify emotional regulation.

The HPA-axis operates as a negative feedback system in which circulating glucocorticoids feedback onto the hippocampus, hypothalamus and the anterior pituitary (among other neural systems), resulting in an inhibition of ACTH release. Other target sites for binding of circulating glucocorticoids include the medial-basal hypothalamus (Dallman et al., 1987) and the hippocampus (McEwen, 1982). Uptake of adrenocorticoids is achieved via two distinct types of glucocorticoid receptors, the mineralcorticoids (Type I) and the glucocorticoids (Type II) (Veldhuis et al., 1982; Beaumont & Fanestil, 1983;

Krozowski & Funder, 1983; Wrange & Yu, 1983; Coirini et al., 1985; Reul & De Kloet, 1985; 1986; McEwen et al., 1986; Reul et al., 1987; Shepard & Funder, 1987). Type I receptors are limited to the septohippocampal circuit, and bind with corticosterone (CORT), cortisol, aldosterone (a mineralcorticoid) and the synthetic glucocorticoid (RU 28362). Type II receptors are more diffusely distributed in the brain and bind with corticosterone, dexamethasone and RU 28362 with high affinity and aldosterone with low affinity.

The type I receptor is generally insensitive to dynamic variations in circulating corticosterone levels, with around 80-90% of receptor sites occupied under basal levels (Reul & De Kloet, 1985; Reul et al., 1987). In contrast, under baseline conditions, only 10-15% of the type II receptors are occupied. Exposure to a stressor increases the hormone-to-receptor signal such that 20 minutes of immobilization results in 75% occupancy of type II receptors. CORT injections which simulate the levels of hormone found during the presence of a stressor also result in about 75% occupancy of receptors. These data have suggested that it is the type II (glucocorticoid) receptor that is responsible for the negative feedback actions of post-stress responses.

As noted earlier, the first empirical evidence which suggested that early handling may have an effect on the HPA system came from another study by Levine (1957). In this study, non-handled Sprague-Dawley rats that received a 20% glucose injection and were subsequently food and water deprived for 24 hours had a significantly greater adrenal weight as compared to early handled subjects (during PND 1-20). This result was

surprising because prior to 1957, adrenal hypertrophy in response to a stressor had been demonstrated to require longer than 24 hours between injection and adrenalectomy.

In another study, Bell (1961) handled 80 Wistar rats on either PND 2-5, 6-9, 10-13 or not at all (non-handled control). Subjects were weaned on PND 21 and reared with same sex and group littermates. On PND 46, individuals from each of the four groups were split into two groups, with one group receiving electric shock in increasing increments until convulsions were produced and the other group receiving no shock. On PND 47, all animals were anesthetized with ether, the right ventricle of the heart was punctured and a sample of blood was obtained. Blood glucose concentration was measured and it was found that the group handled on PND 2-5 did not differ from the non-shocked counterparts. Shocked subjects handled on PND 6-9 and 10-13, as well as the non-handled controls, had elevated levels of glucose as compared to their non-shocked counterparts. This study supported the notion of a critical period for handling during PND 1-5 and implicated the HPA axis due to the observed increase in gluconeogenesis in response to stress.

In 1962, Levine investigated the effects of early handling on circulating CORT levels in response to a noxious stimulus in pups as adults. Forty eight male Sprague-Dawley albino rats were early handled during PND 1-21 (by removing the pup from the nest for 5 minutes). Subjects were weaned and housed 6-8 per cage until PND 60. On PND 60, subjects were randomly assigned to six groups (i.e., time of decapitation post onset of stressor: 0, 15, 30, 60, 300, 900 seconds) and housed in individual cages for ten days (until PND 70). Assignment was such that there were four subjects from the handled

and four subjects from the non-handled groups present in each of the six groups tested post decapitation.

On PND 70, animals experienced a noxious stimulus (electrical shock) and were then decapitated at the pre-assigned times. Blood was collected in a heparinized beaker and assayed for plasma corticosterone. Levine found that handled animals had significantly elevated levels of plasma corticosterone from as early as 15 seconds post shock until 900 seconds. In contrast, non-handled controls only had a significant increase in steroid levels 300 seconds after the onset of shock. Thus, for these data, it seems that infantile handling makes an individual more responsive to stress.

The next important line of evidence came from the study by Hess et al. (1969) described previously. Recall that they found that individuals who were handled at different temperatures (at least until 57 minutes) had an initial increase in plasma corticosterone levels followed by a significant decrease across time. This study, in conjunction with Levine (1962), suggests that early handled subjects show a sharp increase in corticosterone secretion post stressor followed by a rapid decline in corticosterone concentration.

These changes in HPA axis reactivity are not a consequence of changes in adrenal sensitivity (Grota, 1975; Meaney et al., 1989b), pituitary sensitivity to CRH (Meaney et al. 1985a; 1992) or in baseline circulating corticosterone levels (Meaney et al., 1992). Furthermore, Meaney et al. (1989b; 1992) have found that handled and non-handled adults do not differ in baseline levels of corticosterone across their diurnal cycle. Thus,

the evidence suggests that response to a stressor is the differentiating factor between handled and non-handled groups.

- ***The use of a Non-Handled Control Group***

One issue that has remained a theme in the early handled literature is the use of a non-handled control group (Pryce & Feldon, 2003). Early handling requires manipulation of the dam-offspring dyad while the non-handled control group does not. Thus, it seems logical to refer to the former as the experimental group, and the latter as the control group. An alternative interpretation is that in order for rats to display a behavioral profile that is characteristic of the laboratory rat, some minimal amount of stimulation is necessary. That is, non-handled controls actually represent a deprived treatment group while the early handled groups are more similar to animals that receive standard animal husbandry that involves handling in order to provide clean cages. Thus, it is argued that early handled animals are actually similar to typical laboratory rats (Levine, 2000). The fundamental problem with this criticism is that it is hard to define what is typical for a laboratory rat (Levine, 2002). Animal facilities are numerous and spread geographically. Frequency of husbandry procedures, as well as the husbandry procedures themselves, room temperature and humidity all vary and represent possible confounds in any given study. Disputes between veterinarians, the Institutional Animal Care and Use Committee (IACUC) and the researcher as to what constitutes acceptable animal care are not uncommon and may also contribute to variations in methodologies for a given paradigm across laboratories.

Nevertheless, there is evidence which supports the criticism. For example, consider the phenomenon of latent inhibition (LI). Latent inhibition consists of the “retardation in the classical conditioning response of a neutral stimulus to an unconditional stimulus as a consequence of its prior non-reinforced pre-exposure” (Pryce & Feldon, 2003). Latent inhibition is a ubiquitous phenomenon, and some have argued that it is adaptive because it permits an organism to ignore irrelevant environmental information. What is interesting is that several studies have reported the absence of latent inhibition in non-handled controls (Weiner et al., 1985; Weiner, Feldon & Ziv-Harris, 1987; Feldon & Weiner, 1988; Levine, 2002). Thus, in models where the dependent measure is a ubiquitous cognitive phenomenon (i.e., latent inhibition), there is evidence which suggests that the NH group represents the treatment condition.

Rather than trying to define an appropriate control group, Levine (2002) proposes that we conceptualize “control” groups as “comparison” groups since there is no “normal” lab situation. The laboratory rat and the laboratory environment are all artifacts that are created by the experimenter. Thus, it is difficult to determine without adequate comparative research whether the patterns exhibited in the laboratory reflect patterns exhibited in the natural setting. It is likely that in the natural setting, litters experience several “trauma-like” situations (e.g., dam-pup separation as a consequence of dam foraging) that would be unlikely to occur in the lab. Moreover, it is likely that laboratory breeding situations have selected, inadvertently, for patterns of behavior that are relatively rare in the natural setting. Nevertheless, laboratory research has demonstrated that early handling is a robust phenomenon when the comparison group is non-handled.

The insights into the behavior, physiology and neuroanatomy of the laboratory rat derived as a consequence of this comparison all contribute to knowledge about the relationships among physiology, anatomy, and behavior, and therefore give value to this paradigm.

- *Litter Effects*

One problem that has not been addressed in the comparison of EH to NH groups may stem from the experimental design and the use of statistics in exploring these effects of these treatments. In the majority of the studies described previously, the individuals within each group were littermates. The use of multiple littermates within each group has the advantage of decreasing the number of subjects needed (e.g., 6 litters to produce 50-60 subjects vs. 25-30 litters to produce the same number of unrelated individuals per sex and group).

Studies which do not account for the nesting of litters within groups, e.g., the use of Analysis of Variance (ANOVA) without including a variable identifying litter association, enhance the probability of obtaining a significant effect of treatment (a false positive result) (Chapman & Stern, 1979; Denenberg, 1977; Spear & File; 1996). Denenberg (1977) suggests that if multiple littermates are used across each group and an ANOVA is the statistic used, one should include a factor of litter against which a F value can be calculated. Failure to replicate results of studies using littermates nested within group may be a consequence of the high degree of relatedness of individuals within group.

- *The Stress Hyporesponsive Period (SHRP) – The Critical Period Re-visited*

As previously outlined, maximal effects of early handling are observed when treatment occurs during PND 1-7. The first 3 postnatal weeks of the altricial rat are characterized by a high degree of brain plasticity and endocrine patterns that are not observed in the adult animal. The experience of a stressor in the adult rat is associated with the release of CRF from the hypothalamus, ACTH from the pituitary, and CORT from the adrenals. Inhibition of the stress response occurs via negative feedback of glucocorticoids on the brain.

The infant rat can respond to a stressor via upregulation of ACTH and CORT secretions between birth and PND 2 (McCormick, Smythe & Beers, 1994; Zarrow et al., 1967). After PND 2, basal CORT levels decrease and pups show a decreased responsiveness to stressors (Levine, 1994; Sapolsky & Meaney, 1986; Schapiro, 1962). This period of hyporesponsiveness lasts between PND 2-14 and is known as the stress hyporesponsive period (SHRP). Some have argued that as the developing brain is sensitive to damage during this period, the SHRP may have an adaptive purpose of protecting the brain from high circulating CORT levels (Meaney et al., 1991a; Sapolsky & Meaney, 1986).

It is worth noting that the critical period which has been ascribed to early handling (PND1-7) overlaps with both the period when the pup has been demonstrated to be able to respond to a stressor *and* the period when the pup shows a diminished responding to a stressor. Importantly, pups separated from the dam during early handling show an increase in ACTH and CORT (Kuhn, Pauk, & Schanberg, 1990). Early handling

therefore appears to extend the “window” of responsiveness to stressors in the infant pup and this may be mediated, in part, by maternal behavior post reunion. Thus, the critical period via which early handling “produces” its maximal effects are limited inasmuch as our current understanding of the phenomenon itself, as well as the incorporation of time as a relevant aspect of the manifestation of this phenomenon. While it is not within the scope of this review to specify the problems with the ascription of a critical period to a given phenomenon, it is worth acknowledging that at least in the field of psychology, it is often found that “shifting ‘age’ from the description of the phenomenon to its explanation creates the illusion that a difference in plasticity has been discovered” (c.f. Michel & Tyler, 2005).

- ***Transgenerational or “Grandmother” Effects***

Maternal behavior exhibited by the dam to female pups as a consequence of early handling has been demonstrated to affect the way the adult female offspring treat their own offspring. In such transgenerational or “grandmother” effects, male and female offspring of dams handled as pups display a characteristic EH behavioral profile, even though these offspring were untreated as pups (Denenberg & Whimby, 1963).

Denenberg & Whimby (1963) compared two groups of rats that were either handled or non-handled for 3 minutes daily during PND 1-20. As adults, EH and NH female offspring were bred and gave birth to their own litters. Half of the pups from the second generation litter were reared with their biological mother and the other half cross fostered to a foster mother with an early experience opposite to that of their biological

mother (i.e., a NH foster mother if the pup's biological mother was EH or a EH foster mother if the pup's biological mother was NH).

Second generation offspring were tested over four days in an open field on PND 50. Males reared by EH dams had higher rates of defecation as compared to those reared by NH dams. Interestingly, males born to NH dams but raised by EH dams were the most active in the open field across four days of testing. The second highest activity levels were displayed by males reared by non-fostered EH dams. No differences were observed between any of the other groups.

Thus, early handling of rat pups during the nursing phase affected the development of the daughters such that when they gave birth to their own offspring as adults, patterns of interaction of the dam with male offspring were different from female offspring. This difference in maternal behavior resulted in male offspring with a blunted emotional profile.

Fleming et al. (2002) have also reported that rat dams exhibit patterns of maternal care similar to that of their own mothers and grandmothers. Fleming et al. (1979) had previously reported that either bulbectomy or disruption of functioning of the olfactory bulb reduces the frequency of pup-directed licking behavior. Thus, in the study by Fleming et al. (2002), dams were either bulbectomized by aspiration or sham operated prior to parturition. Dams in each group reared their young until weaning and maternal behaviors were observed (e.g., retrieving, crouching, licking). Analysis of maternal behaviors indicated that only licking behavior was affected. Bulbectomized dams licked their pups (body and anogenital regions) less than sham operated dams.

Subsequent to weaning, pups were housed in same sex dyads. One female pup from each litter was randomly selected from the sham operated and from the bulbectomized groups, respectively, to be bred and observed for pup-directed maternal behavior. Note, neither bulbectomy nor a sham operation was performed in this generation. In this second generation, the same patterns of licking were observed by daughters of mothers in each group. That is, daughters of bulbectomized dams licked their pups less (body and anogenital regions) than daughters of sham operated dams.

Furthermore, even though each generation displayed similar patterns of maternal behavior, in the first generation the patterns of maternal behavior were a consequence of an intervention affecting the mother, while in the second generation the decreased licking appeared to be a consequence of not experiencing certain patterns of licking during development. Thus, the effects of early infantile experience in the form of early handling may have pervasive effects on offspring and future generations of offspring, as patterns of maternal behavior post reunion have been demonstrated to be affected by this manipulation.

- *Sex Differences*

There is some evidence which suggests that the effects of early handling may be differentially manifest in male and female pups. In my review of the literature, Wells (1976) was the first researcher to investigate whether males and females differ in their response to early handling. Hooded rat pups, with an equal sample number across groups (actual n/group was not reported in the paper), were either handled or non-handled and

their rearing behavior in a home cage (Experiment 1); latency to leave the home cage via an open metal grid door on the lid (Experiment 2); and response to novel stimuli (Experiment 3) were assessed as adults.

In Experiment 1, it was demonstrated that non-handled males exhibited longer latencies to rear across trials, while non-handled females, handled males and handled females all exhibited a decline in their latency to rear across trials. In experiment 2, they found that handled subjects and female subjects had a lower latency to leave the home cage across trials. No interaction between sex and handling condition was observed. In experiment 3, they found that non-handled subjects had a reduced latency to approach and spent less time investigating a novel object. It is also worth noting that greater variability was observed in females than males across trial, irrespective of handling condition. The author suggests that this may in part be a consequence of estrous cycling.

Wells (1976) concludes that the only differences between males and females are in part determined by the nature of the testing environment. That is, based on the results of this study, they suggest that the greater the locomotor activity component in the test, the more likely that a straightforward handling and sex difference will be manifest. They also assert that it may be an oversimplification to state that males and females respond differentially to early handling.

Levine's group also systematically evaluated whether a sex difference exists in the early handling paradigms (Weinberg, Krahn & Levine, 1978). This hypothesis was stimulated in part by the observation that early handling reduced the effects of shock-induced fighting to a greater extent in Sprague-Dawley females than in males (Erksine,

Stern & Levine, 1975), and that early handled Sprague-Dawley females showed a lower elevation of plasma corticosterone levels in response to rapid avoidance training than males (Weinberg & Levine, 1977).

In their study, Weinberg et al. (1978) bred 50 litters of Sprague-Dawley females crossed with Long Evans male rats that were either handled or non-handled. Pups were removed daily during PND 2-17 for 3 minutes and individually housed in 6x6x5 cm compartments. Subjects were weaned on PND 22, and four males and four females were selected from each litter (n=400) and housed in hanging cages until PND 60. On PND 60, 24 males and 24 females from each group (handled and non-handled) were randomly selected from the population of 400 and re-housed in 28x24x15cm pans. Subjects were tested at an unspecified age on a hole-board which permitted assessment of exploration (the authors' index of "emotionality") independent of ambulation. They found that females head-dipped for a longer duration than males, and that the duration of head-dips did not decrease for females across days, as it did for males.

While it is not within the scope of this paper to outline every study which has found a sex difference in the effects of early handling, the general conclusion is that males tend to be more affected by the manipulation of early handling than females (c.f., shock induced fighting - Erksine, Stern & Levine, 1975; latent inhibition - Peters et al., 1991; Weiner et al, 1985, 1987; active avoidance – Weinberg & Levine, 1977; and exploration: Weinberg et al., 1978). However, as Wells (1976) has pointed out, it is not sufficient to ascribe a general sex difference but rather we need to specify the causal

pathway via which being male or female may affect phenotypic outcome of early handling.

- ***Contextualizing the Causal pathway in the Early Handling Phenomena***

In summary, there appears to be a critical period via which the effects of early handling are maximal (i.e., “produces” adults with a blunted emotional profile). The temperature of the pups as well as the post reunion response of the mother both seem to be necessary for the manifestation of this characteristic behavioral profile. Furthermore, there is some evidence that early handling may differentially affect male and female rat pups. In this section, I will attempt to link these variables together because the review of the literature reveals that no one study has acknowledged the contributions of all of these variables or systematically assessed how these variables may contribute to the manifestation of the “characteristic” early handled behavioral phenotype.

Female mammals have an internal gestation, and are generally primarily responsible for the nourishment of young. This makes them indispensable in the rearing of young, particularly if the young are altricial at birth. Offspring of the order *Rodentia* are altricial at birth and thus are dependent on the mother for nourishment. The first three postpartum weeks are of marked importance in the species *Rattus* (i.e., the rat), with levels of maternal behavior increasing dramatically post-partum and then gradually waning as weaning approaches (generally in the third week of life).

A few days before parturition, an increase in nest-building is generally observed (Kinder, 1927; Rosenblatt & Siegel, 1975). Immediately after birth, licking, retrieval and

nursing (pup-directed maternal behavior) are all observed (Rosenblatt & Lehrman, 1963). As rat pups are altricial, these behaviors are necessary for survival. One key role of the dam postpartum is the maintenance of thermoregulation. At birth, muscular sources of thermoregulation in the rat pup (e.g., locomotion, shivering and piloerection) are not mature (Farrell & Alberts, 2007). For example, Taylor (1960) has found that shivering does not contribute to the thermoregulatory process until about the third week of life (generally when pups are weaned). This, in conjunction with the lack of fur, lack of subcutaneous white adipose tissue and a large surface to volume ratio, leads to rapid heat loss when the pup is removed from the natal nest or placed in a cool environment (Conklin & Heggeness, 1971; Hull, 1973; Malik & Fewell, 2003).

While muscular sources of thermoregulation are not available in rat pups, brown adipose tissue (BAT), the metabolism of which is a means of heat production, is present. Hull (1973) conceptualized BAT as an infant rat's only source of non-shivering thermogenesis. BAT is present in the newborn rat, is distributed near vital organs and blood supplies (Blumberg, 2001; Hull, 1973; Smith, 1964), and is thermogenic, with its metabolism capable of warming nearby blood supplies and organs.

Even though pups are incapable of thermogenesis, behavioral thermoregulation is possible individually (Hoffman, Flory & Alberts (1999) or as a litter (Alberts, 1978, 2007). Huddling in a litter reduces the surface area to mass ratio, thereby leading to a reduction in heat loss. Huddling has been demonstrated to be a dynamic structure where the behavior and physiology of individual pups contribute to the overall thermoregulatory state of the litter (Alberts, 1978, 2007). When pups are exposed to cool temperatures, the

huddle reorganizes such that individuals in the huddle constantly rotate from the top to the bottom of the huddle in a convection-like pattern until thermo-neutrality occurs (~34-36°C) (Alberts, 1978).

Thus, as a litter, behavioral thermoregulation is possible immediately post partum while physiological thermoregulation is not. In terms of the early handling paradigm, these ecologically valid observations of behavioral thermoregulation are rendered moot as all of the early handling procedures described previously generally involved separation of the dam from the litter and then the littermates from each other for about 15 minutes daily during the PND 1-7.

When an infant rat is isolated from the dam and its littermates, a behavioral and a physiological response is mounted (Blumberg & Alberts, 1990). The physiological component involves increasing the production of metabolic heat via the breakdown of BAT (Spiers & Adair, 1986; Taylor, 1960). The behavioral component involves the production of ultrasonic vocalization (USVs) (Allin & Banks, 1971; Noirot, 1972). It should be noted that in this context, ultrasonic refers to frequencies of sound outside of the range of the hearing of humans. Blumberg and Alberts (1990) found that when pups were exposed to cold (26-29 °C) BAT metabolism, oxygen consumption and respiratory rate increased. Additionally, they found that *continuous* production of USVs occurred around 10 minutes from the time of exposure to the temperature drop, with an increase in USVs production observed as early as 1 minute, and a statistically significant increase in USVs production observed at 2 minutes. Notably, when BAT was observed to be producing heat, USVs concurrently increased. Thus, separation of the pup from the dam

and the littermates from each other at room temperature (as is commonly done in the early handling manipulation), appear to represent a distressful thermoregulation event. Blumberg and Alberts (1990) note that ultrasonic pulses occur in tandem with the increased durations of expiratory movements of the lungs. This phenomenon is known as laryngeal braking (Davis & Bureau, 1987; England et al., 1985; Gautier et al., 1973) and is thought to improve gas exchange by increasing lung volume and recruitment of new alveoli.

Although pup USVs may serve a communication function, the evidence suggests that the production of USVs as a consequence of early handling can be a byproduct of the mechanisms used for adequate gas exchange during exposure to cold (Blumberg & Alberts, 1990). USVs need not have evolved as a communicatory device for maternal retrieval but instead may have been co-opted (an exaptation) for maternal retrieval. In other words, sound is produced when the larynx is used for laryngeal braking. If separation from the natal nest occurs with some frequency in the natural habitat, then mothers can increase their reproductive success by using USVs to retrieve pups that are isolated from the nest. There is some evidence which suggests that secondary adaptations may have arisen and been maintained as they led to greater reproductive success. For example, Brown (1973a, 1973b) has found that the auditory sensitivity of many rodents may have been modified such that they now contain two sensitivity peaks. One of these peaks corresponds to the vocalization frequency of conspecific young.

Farrell and Alberts (2002a, 2002b) were the first to systematically demonstrate that maternal responsiveness in the Norway rat was at least in part under the control of

USVs. The term maternal responsiveness in this context is used to denote “an internal state or condition that is manifest by the expression of maternal behavior in the presence of cues from young” (Rosenblatt, 1965). With this definition, it becomes a hypothetical construct and thus is not directly observable. Farrell and Alberts (2002a) exposed virgin, pregnant, and parturient dams to pups that were warm and silent (baseline). Subsequently, they lowered the temperature of the stimulus pup and observed the reaction of the dam while the pup was vocalizing. The dependent measure was the amount of time the dam spent oriented toward the stimulus pup. It should be noted that the female was never in contact with the pup but was able to orient toward the pup via a mesh covered hole near to the pup. Thus, the authors used this proximal orientation response as their measure of maternal responsiveness. They found that maternal responsiveness increased during the first week post partum and declined thereafter until the third week of rearing (i.e., weaning). Thus, during the critical period that has been ascribed for the maximal effects of early handling (i.e., PND 1-7), maternal responsiveness in the Norway rat appears to be mediated by USVs.

This study is important because it highlights the role of USVs in the absence of other extraneous variables. That is, the role of USVs in eliciting maternal responsiveness in the absence of other possible cues was investigated. However, as mentioned before, maternal responsiveness is a hypothetical construct – other studies have described licking, retrieval and nursing behavior as maternal responsiveness (c.f., Rosenblatt & Lehrman, 1963). Thus, the role of USVs in the presence of other cues from the natal nest

must be explored in order to specify whether USVs is a necessary condition for the onset of maternal responsiveness.

In the companion article to Farrell & Alberts (2002a), the role of pup odor in eliciting maternal responsiveness was investigated (Farrell & Alberts, 2002b). In Experiment 1, they found that playback of USVs to both parturient and virgin females resulted in equal increases in the levels of maternal responsiveness. Parturient dams only showed heightened levels of maternal responsiveness when acoustic playback occurred in the presence of a warm non-vocalizing pup. Thus, non-acoustic cues seem to be required for increased maternal responsiveness. In Experiment 2, the authors investigated whether the odor of the pups was a necessary variable in the onset of maternal responsiveness. To this end, the authors investigated whether anosmia would alter mother rats' responsiveness to a live pup. Mothers were assigned to either a saline or a ZnSO₄ (anosmic) group. Pre-infusion, both saline and ZnSO₄ groups displayed an 80-90% accurate retrieval rate. Post- infusion, they found that anosmic mother rats showed a general failure to retrieve vocalizing pups while the saline group retained a similarly high level of retrieval. Thus, it appears that olfactory cues co-act with auditory perception to yield maternal responsiveness.

In Experiment 3, the authors investigated whether mothers used non-acoustic elements of the pup stimulus as a directional cue when responding to USVs. To this end, mother rats were presented with playback USVs from a speaker in the presence of a warm silent pup. Groups were either spatially matched (control group, under the speaker) or spatially disparate (treatment group, on the wall opposite the speaker). They

found that the control group displayed higher levels of maternal responsiveness than the treatment group. Thus, it appears that pup odor interacts with USVs in order to facilitate maternal responsiveness.

As demonstrated before, the effects of early handling are manifest only when there is a corresponding decline in pup temperature. Thus, when pups are individually separated from the littermates for fifteen minutes daily during PND 1-7, the inability to effectively thermoregulate their temperature may result in the production of USVs. Therefore, upon reuniting the dam with the pups, maternal responsiveness will increase. The question now becomes, how do changes in maternal responsiveness correspond to differences in the “emotional” profile of pups as adults?

Several lines of evidence suggest that the effects of handling are mediated by the changes in the tactile stimulation provided by the dam during the first week of life (Smotherman, 1983). D’Amato et al. (1998) has found that treatment of the dam with Chlordiazepoxide (an anxiolytic and skeletal muscle relaxant) prevents the compensatory increase in licking and grooming following early handling and “produces” offspring who are similar in their behavioral profile to non handled controls. Rosenberg, Deneberg and Zarrow (1970) found a significant correlation between levels of maternal care (as assessed by a 7-point rating scale looking at lying upon, huddling with, retrieving, and licking or grooming) and secretion of corticosterone in response to stress. Thus, the evidence suggests that increased levels of maternal care are necessary to program a resilient “emotional” profile of pups as adults.

Perhaps the best evidence assessing the question of “How do increased levels of maternal responsiveness lead to a more resilient “emotional” profile?” can be obtained from examining natural variations in maternal behavior across individuals. Champagne et al. (2003), in a quantitative analysis of maternal behavior in Long Evans rats, found that variability in maternal care is associated with variations in the frequency of Licking/Grooming (LG) and Arch Backed Nursing (ABN). Also, high correlations were observed between these two discrete behaviors such that dams that showed high levels of Licking/Grooming also showed high levels of Arch Backed Nursing (Caldji et al., 1998). Furthermore, dams that were classified as high LG-ABN were not significantly different from low LG-ABN in other maternal behaviors, such as time spent in contact with pups or in nursing pups.

Interestingly, offspring raised by high LG-ABN dams show a behavioral profile that is similar to offspring who are handled (Liu et al., 1997). That is, they have decreased HPA activation in response to stress, and behaviorally are “less fearful” when exploring novel environments. Like handling, these changes persist into the animal’s adult life, and changes in gene expression and molecular substrates (outlined below) parallel those seen in offspring exposed to high LG-ABN. Finally, the observation that differences in LG-ABN are confined to the same critical period as that of handling (i.e., PND 1-7) provides evidence that LG-ABN and early handling share a similar neurodevelopmental pathway.

Weaver et al. (2004) provides a comprehensive review of the molecular effects of handling. As highlighted previously, handling of pups individually during PND 1-7

involves a transient drop in pup temperature. This change in body temperature leads to the activation of the Hypothalamic-Pituitary-Thyroid (HPT) axis (Meaney et al., 1994). This stimulates an increase in circulating levels of thyroxine (T_4) and triiodothyronine (T_3), and in adults, affects the binding capacity but not the receptor density of glucocorticoid receptors in the hypothalamus and the pituitary (Meaney, Aitken & Sapolsky, 1987). Furthermore, the administration of propylthiouracil (PTU), a thyroid hormone synthesis inhibitor, blocks the effects of handling on glucocorticoid receptor (GR) binding. T_3 and T_4 plasma levels in handled animals parallel those found in animals exposed to high levels of LG-ABN. Kaffman and Meaney (2007) also suggest that handling or LG-ABN induces thyroid metabolism of T_4 to T_3 and may affect hippocampal GR density. This assumption is derived from a previous study which found that administration of either T_3 or T_4 to pups on PND 1, 2 and 4 is associated with increased levels of GRs in the hippocampus (Meaney, Aitken & Sapolsky, 1987).

There is some evidence which suggests that the effect of thyroid hormones on the GR system is mediated by the ability of thyroid hormones to increase serotonin (5-HT) turnover (as measured by the ratio of 5-Hydroxyindoleacetic acid (5-HIAA) to 5-HT) (Mitchell, Iny & Meaney, 1990). Handling has also been demonstrated to increase 5-HT turnover (Mitchell, Iny & Meaney, 1990). Increased 5-HT turnover chronically activates cyclic adenosine monophosphate (cAMP) pathways in hippocampal cells via the 5-HT₇ receptors pathway. Elevation in the levels of cAMP signaling is associated with the increased expression of Nerve Growth Factor Inducible-A (NGFI-A). NGFI-A binds to the promoter region of the GR Deoxyribonucleic acid (DNA) sequence which in turn

activates transcription in handled animals (Meaney et al., 2000). Thus, handling may lead to upregulation of GR expression in the hippocampus. Lesioning of raphe 5-HT with 5,7-dihydroxytryptamine (5,7-DHT) reduces ascending serotonergic input into the hippocampus and leads to decreased hippocampal GR receptor density (Mitchell, Iny & Meaney, 1990).

In vivo experiments demonstrating that handling modifies NGFI-A binding to the GR promoter region have not been performed. However, Weaver et al. (2004) have demonstrated *in vivo* that offspring of high LG-ABN dams show increased binding of NGFI-A to the GR promoter region as compared to low LG-ABN subjects. Given the postulated homology of the neurodevelopmental pathway of early handled offspring and offspring reared by high LG-ABN dams, it may be possible that early handled offspring show increased binding of NGFI-A to the GR promoter region.

Interestingly, while high levels of NGFI-A are associated with offspring reared by high LG-ABN dams, no difference in NGFI-A levels are observed in offspring reared by either high LG-ABN dams or low LG-ABN as adults. Given that the effects of handling and high LG-ABN nursing persists through adulthood, the question now becomes what maintains the ability of NGFI-A to bind more efficiently in offspring reared by high LG-ABN dams and how does maternal care mediate these effects?

One line of research which investigates this question and has received empirical support involves DNA methylation. While it is beyond the scope of this review to specify the various details of DNA methylation, a brief introduction is necessary. DNA methylation involves enzymes known as DNA methyltransferases (DNMT). These

enzymes “scan” the DNA for cytosine-phosphate-guanine (CpG) sites, and are capable of transferring a methyl group to this molecule to form 5-methylcytosine. The addition of a methyl group compacts DNA around its histone backbone, making it less accessible for gene expression. Conversely, histone acetyltransferases (HAT) can add an acetyl group to specific histones which makes DNA more accessible, and therefore more accessible for transcription. Histone deacetylases (HDAC) can remove the acetyl group added by HAT, thereby rendering DNA less accessible for transcription. While DNA methylation is thought to be an irreversible reaction in adult post-mitotic cells, Weaver et al., (2004) have demonstrated that it is possible to induce replication-independent demethylation of methylated genes by increasing histone acetylation using the HDAC inhibitor trichostatin A (TSA). Activation of chromatin through HDAC inhibition has been shown to trigger DNA demethylation (Weaver et al., 2004).

Longitudinal assessment of the GR promoter region methylation state reveals that many CpG sites, including two that bind NGFI-A, are hypermethylated (Weaver et al., 2004). Exposure to high levels of LG-ABN during PND 1-5 is associated with a reduction of methylation at the GR promoter region, and this persists through adulthood (Weaver et al., 2004). Furthermore, there is some evidence of promoter remodeling, as offspring reared by high LG-ABN dams show 3-4 times greater binding affinity to the GR promoter region than offspring reared by low LG-ABN dams (given comparable levels of NGFI-A) (Weaver et al., 2004). Thus, high levels of LG-ABN, and likely handling, lead to a more efficient transcription of GRs in the hippocampus in offspring as adults.

- *The Role of Sex Differences*

Thus far, I have outlined a possible causal pathway that may explain how the effects of early handling can be manifest in offspring as adults. A few loose ends still remain: sex differences and further discussion of an adequate control group. These issues can again be addressed using studies that were initially observational and later experimental. Gubernick and Alberts (1983) describe that, in rats, the frequency of LG-ABN subsides over the first ten postnatal days. Licking behavior, particularly in the anogenital region (i.e., anogenital licking (AGL)), stimulates urination and defecation which are not under “voluntary” control by the pups during the first 5-7 postnatal days. Thus, AGL behavior is essential for the survival of the pup. Furthermore, AGL stimulated urination and defecation permits the dam to recycle about 70% of the nutrients that are lost through nursing (Gubernick & Alberts, 1983).

Moore and Morelli (1979) have demonstrated that Long-Evans rat dams exhibit higher levels of AGL for *each* male pup as compared to *each* female pup in a litter. This naturally occurring difference in licking was demonstrated to be a consequence of secretions from the preputial gland (Moore, 1982; Moore & Chadwick-Dias, 1986). These secretions act as an attractant to the dam, and there is some evidence that the metabolite that mediates this effect is dodecyl propionate (Brouette-Lahlou, 1991). Furthermore, Moore (1982) has demonstrated that normal perinatal secretions of testosterone from the male gonads maintain the activity of the preputial gland postnatally in male pups, while activity of the preputial gland rapidly decreases postnatally in female pups.

Interestingly, Moore and Chadwick-Dias (1986) have found that males respond more rapidly to AGL by exhibiting a leg extension reflex (LER). Briefly, the LER is elicited by cutaneous stimulation of the perigenital region and resembles an adult lordosis response characteristic of females in estrus (Williams & Blaustein, 1988). The characteristic pattern of responding to the dam by LER is faster in male than female pups and is followed by immobility. This pattern of responding in male pups may contribute to the facilitation of AGL by the dam. This finding, in conjunction with the identification of an attractant secreted by the preputial gland, may explain the differential amount of maternal AGL observed between male and female pups.

Some evidence suggests that male rat pups also vocalize more vigorously than females (Naito & Tonoue, 1987). Briefly, male and female Wistar rat pups were individually removed from the natal nest from PND 0-18, placed in a beaker at room temperature, and the emitted ultrasound was recorded. Subjects were counterbalanced across sex and litter (c.f., Naito & Tanoue, 1987). It should be noted that this procedure is typical of the early handling procedure even though it was not formally described as such in the study. The authors found that female USVs tended to be shorter in duration and less variable as compared to males. Males emitted more vigorous USVs than females when the litter composition was mixed. However, no sex differences between USVs were observed between litters that were comprised of only of males or only of females. So, this sex differences may relate only to mixed sex litters.

As mentioned before, rat pups are relatively unable to voluntarily urinate and defecate during the first 5-7 postnatal days without stimulation of the perigenital region.

The focus of AGL on the perigential region has been shown to be in part under the control of the attractant dodecyl propionate which is secreted from the preputial glands. Brouette-Lahlou, Vernet-Maury and Godinot (1992) were the first to demonstrate that the vomeronasal organ and not the olfactory epithelium is necessary for detection of the secretions from the preputial gland. Interestingly, they observed only a 25% mortality rate in pups that were reared by dams whose vomeronasal organ was removed and a 35% mortality rate in preputialectomized pups.

If urination and defecation are necessary for pup survival and AGL is mediated by the ability to detect the attractant dodecyl propionate from the preputial gland, then AGL must be under multiple and redundant sensory control (Beach & Jaynes, 1956), as removal of the vomeronasal organ results only in a 25% mortality rate and preputialectomy results in a 35% mortality rate. Thus, the detection of dodecyl propionate may only be relevant for the manifestation of sex differences in AGL. In a follow up study, Brouette-Lahlou et al. (1992) investigated the role of USVs in stimulating AGL by dams in preputialectomized pups. Of note, the dams used in this study were primiparous. In Experiment 1, they found that pup USVs preceded bouts of AGL in both the preputialectomized and the control subjects. However, preputialectomized pups were significantly more likely to continue to produce USVs after an AGL bout than control pups. In Experiment 2, they found that dams were more likely to sniff (but not ingest) pieces of filter paper spiked with 10ng of dodecyl propionate. However, when presentation of the filter paper spiked with dodecyl propionate was coupled with

playback of pup USVs, dams were more likely to ingest the pieces of filter paper than controls.

The study by Brouette-Lahlou et al. (1992), like the one by Farrell and Alberts (2002a, 2002b), suggests that USVs may serve to induce maternal responsiveness (operationally defined here as bouts of AGL). Secretions from the preputial gland may serve to regulate AGL. While these USVs and secretions from the preputial gland act in a complementary fashion, they are able to independently stimulate AGL. Thus, AGL can be thought of as being under multiple and redundant sensory control.

Dodecyl propionate, a secretion from the preputial gland, acts as a metabolite attractant to the dam. The production of this attractant stimulates licking of the perigenital region of the pup (particularly the male pup) by the dam, thus facilitating urination and defecation of the pup. As recycling of electrolytes and nutrients by the dam occurs via ingestion of urine and feces, it is unclear whether AGL in the presence of USVs stimulates this process. The study by Brouette-Lahlou et al. (1992) only suggests that general ingestive behavior occurs via the complementary action of these two systems.

In the literature, the general conclusion is that males tend to be more affected by the manipulation of early handling than females (c.f., shock induced fighting – Erksine et al., 1975; latent inhibition - Peters et al., 1991; Weiner et al, 1985, 1987; active avoidance – Weinberg & Levine, 1977; and exploration - Weinberg et al., 1978). I have provided evidence that the core body temperature reduction in pups as a consequence of early handling stimulates the production of USVs (a byproduct of laryngeal braking), and there is also some evidence which suggests that male rats produce more vigorous and longer

duration USVs than females. USVs in turn facilitate an increase in maternal responsiveness and in particular, facilitate an increase in LG-ABN behavior. Furthermore, AGL has been shown to be differentially manifest in male and female pups, and the general upregulation of LG-ABN has profound influences on the organization of the nervous system. Thus, one mechanism via which sex differences due to early handling may emerge may be a consequence of different patterns of maternal care elicited by (via USVs) and exhibited (via AGL) to male and female pups.

- ***The Correct Control Group***

In a previous section, the use of different control groups across studies was discussed. In general, studies investigating the effects of early handling have used a non handled control as the reference group. More recent studies addressing the notion (Levine, 1960; Pryce & Feldon, 2003) that NH controls represent an impoverished treatment condition have begun to use an Animal Facility Reared (AFR) control group in the investigation of early handling. Some studies indicate that the AFR rats are different from NH rats when measured on latent inhibition as adults (Weiner et al., 1985; Weiner, Feldon & Ziv-Harris, 1987; Feldon & Weiner, 1988; Levine, 2002). Other studies report no differences between AFR and EH rats, e.g., ambulation in a 5 minute test (Meerlo et al., 1999); ambulation in a five minute open field across four days of testing (Roy & Chapillion, 2004).

Very few researchers have directly compared EH, NH and AFR groups in one study. Lehmann and Feldon (2000) report that AFR, EH and NH groups do not differ in

terms of HPA reactivity and cognitive performance when tested at PND 540 (18 months). Furthermore, no study has investigated why a simple innocuous experience as standard animal husbandry (AFR) may produce long term behavioral differences similar to EH groups in offspring as adults. Thus, the research suggests that early handling in rats attenuates behavioral responses to novelty in offspring as adults when the comparison group is a non-handled one. It is unclear, other than in an operational sense, what differentiates the AFR and NH control group. That is, AFR involves handling 1-2 times per week during standard animal husbandry depending on laboratory schedules, and in some studies, movement of personnel in and out of the room (which does not occur with the NH group).

In summary, the effects of EH when compared to a NH group are robust and appear to be under multiple sensory and redundant control in the rat, regardless of strain (Lehmann & Feldon, 2000). However, many of the differences observed in the EH group are removed by the AFR control condition. The causal pathway via which a blunted “emotional” profile emerges in offspring as adults is beginning to be worked out. Studies investigating the role of early handling on later “emotionality” should address the variables identified in this review. These variables include but are not limited to pup temperature post separation, differential production of USVs post reunion across sexes, differential patterns of maternal care exhibited to male and female offspring, litter effects, and the use of appropriate statistical analyses.

Early Handling in Mice

While the preponderance of research into the effects of and the mechanisms behind the phenomenon of early handling has been performed using rats, some work has been done using mice, particularly inbred strains of mice. In my review of this literature, two themes repeat themselves. The first is the notion that “the gene” and “the environment” are separable. Given that inbred mice are genetically identical, then “under the assumption that environmental conditions have been held relatively constant or have varied in only a random fashion, the results of these studies” (i.e., strain differences within mice and differences between mice and rats on the effects of early handling) “have been viewed as demonstrations of a direct genetic influence upon behavior” (Ressler, 1962). This theme prevails in the majority of the research on early handling and may stem from popular notions at that time (e.g., that genes limit the phenotype in any environment (Range of Reaction) and that the environment limits the genetic potential that can be realized).

The second theme that arises is that there is an abrupt change in the control group in the literature looking at the effects of early handling in mice. Initial work investigating the effects of and the mechanisms behind early handling using mice described the effects of this manipulation in reference to a non-handled control group. As in the rat literature, later studies shift the comparison group to an animal facility reared one.

In my literature review, the first study investigating the effects of early handling in mice was performed by Deneneberg and Karas (1959). In this study, litters of rats descended from a Harvard Wistar strain and litters of mice from a C57BL/10Sc strain

were assigned to the following groups with at least two litters of mice and rats per group: 1) handled during PND 1-10; 2) handled during PND 11-20; 3) handled during PND 1-20; or 4) a non-handled group control group. Their handling manipulation consisted of removing the pups from the nest and placement of the pups in a container with sawdust. Subjects were weaned on PND 21 and reared with littermates of the same sex, food and water *ad libitum*. The number of individuals per cage was described as “small”. On PND 64 rats were weighed, isolation housed and then food and water deprived. Mice received the same treatment on PND 54. It was unspecified why isolation and food deprivation were performed on different days for rats versus mice. The number of hours before death occurred was recorded.

Deneneberg and Karas (1959) found that rats handled on PND 1-20 were the heaviest and those that were not handled were the lightest. In addition, the two groups that were handled during the first week of life (i.e., during PND 1-10; groups 1 and 3) weighed significantly more than any of the other groups. Notably, a different relationship between handling in mice and weight gain was observed. They found that those that were handled during the second week of life (i.e., during PND 11-20; groups 2 and 3) weighed significantly more than any of the other groups. In addition, rats that were handled during PND 1-20 or not handled died significantly earlier whereas, in mice, only subjects that were handled during PND 1-20 experienced significantly higher mortality rates. It should be noted that the difference in mortality rates across groups in rats maps discretely with initial weight status while this relationship is less clear in mice. Denenberg and Karas (1959) suggested that the mouse data may be biased since mice have a greater prevalence

of spontaneous death. This initial paper by Denenberg and Karas (1959) was the first paper to suggest that the effects of early handling may be differentially manifest in mice and rats.

The next study investigating the effects of early handling in mice was published eight years later by Thiessen et al. (1967). They stated that the experiment was originally begun with three inbred strains of mice (CBA/J, AJ, and C57BL/6) but due to a high mortality of litters from dams of C57BL/6 and AJ, only CBA/J mice were tested. The authors stated that “handling was much more stressful for these animals, and the interaction between handling and drug toxicity might be quite different” which suggests a gene by environment interaction.

In this study, 15 CBA/J litters were bred and “half” of the litters were assigned to either a handled group or a non-handled group. It remains unclear how 15 litters were “halved”. Pups were weaned on PND 20 and tested on PND 60. Thiessen (1964) had previously noted that exposure to shock or changes in ambient temperature “modify the toxicity of the sympathomimetic drug amphetamine”. Furthermore, he highlighted that exposure to stressors immediately before drug administration have been demonstrated to increase the toxicity of amphetamine, while exposure to stressors “considerably before” drug administration “reduces” the effects of drug toxicity via an unspecified adaptation. As the phenomenon of early handling at that time was conceptualized as an “adaptation to stress” (Levine, 1962); Thiessen (1967) hypothesized that early handling would “offer” some “protective effect” to the toxicity of amphetamine. He found that early handling significantly attenuated the effects of amphetamine toxicity by 18% as compared to the

non-handled control group. Thus, like other studies at the time, early handling appeared in infancy to confer “some resiliency” to pups when tested as adults and when compared to a non-handled control group.

In an extension of the study described earlier by Levine, Chevalier & Korchin (1956), Smith (1967) described the effects of brief periods of handling and exposure to varying intensities of electrical shock (0.2, 0.4, 0.6, 0.8 milliamps (mA) for 90 seconds) during PND 1-10 in C57BL/6J mice on learning behavior. Recall, Levine et al. (1956), reported that Sprague-Dawley rats that were either handled or shocked for 3 minutes daily during PND 1-20 displayed superior learning on a conditioned avoidance task in a shuttle box (rapid avoidance learning) as compared to non-handled controls.

In this study, Smith (1967) randomly assigned 55 litters of C57BL/6J inbred mice to one of six early treatment groups and a later training task. It remains unclear how 55 litters were evenly distributed across 6 treatment conditions. For five of these six groups, early treatment consisted of placement of the pups in an “infant stimulator” for 90 seconds during PND 1-10; where “one group was simply placed in the apparatus for 90 seconds but received no shock, whereas the others received 90 seconds of either 0.2 ma., 0.4 ma., 0.6 ma., or 0.8 ma.” (Smith, 1967). The sixth group served as a non-handled control group. All 55 litters were undisturbed from PND 10 until PND 21 when they were weaned. At weaning, females were removed as subsequent evaluation of treatment effects were done only on males. Males were housed with their littermates and thus were housed with subjects in the same early treatment condition.

On PND 40 subjects were subjected to one day of pre-training in a visual discrimination apparatus. The apparatus consisted of a start box with a sliding door that led to an electrified grid floor. Both the start box and the electrified grid floor were capable of delivering a 0.8 mA shock. The electrified grid floor led to two partitioned corridors (left and right) capable of delivering a 0.5 mA shock. The two corridors led to two doors which exited to and un-electrified the goal box. All four zones of the apparatus were capable of independent administration of electric shock. A 100 watt bulb was placed two feet above the doors and provided illumination of the apparatus.

Pre-training consisted of the following procedure. Subjects were first taught to escape the electrified area of the apparatus. If a subject failed to leave the startbox within 5 seconds it was shocked every 2 seconds for 1 second. The subject then had 25 seconds to escape to the goal via either the left or right corridor, which was ungated during pre-training. Failure to exit the corridor to the goal, resulted in the electrification of all four zones of the apparatus, until the subject proceeded to the goal. An inter-trial interval of 30 seconds was given throughout pre-training. When the subject made three successful exits to the goal without shock (it is unspecified whether they need to be successive), the gate of its last exit was barred, and the corridor in front of it was electrified. The subject was then replaced into the startbox and when the subject exited the apparatus once via the un-electrified corridor, pre-training ceased.

On PND 41 training began. Subjects were randomly assigned to either a spatial, brightness or pattern discrimination condition. For each condition, the exit doors to the goal varied depending on the task.

For spatial discrimination, identical grey doors were used but for brightness discrimination black and white doors were substituted. The pattern discrimination was accomplished using doors with horizontal and vertical stripes that were equated for black and white area (achieved by reducing slightly the width of the vertical stripes) which, therefore, equated the two stimuli for the brightness and luminous flux (Smith, 1967).

In each discrimination condition, both gates were put in place and the negative gate was locked with the corridor to the negative gate electrified. Similar to pre-training, if a subject failed to leave the startbox within 5 seconds it was shocked every 2 seconds for 1 second. If the subject failed to leave its choice area after 25 seconds, a one second shock was administered every 10 seconds. “Subjects were trained on one of three problems: (1) a position response in which either the right or the left door was unlocked, (2) a brightness discrimination with either black or white as the positive stimulus, (3) a pattern discrimination with either horizontal or vertical stripes as the positive stimulus” (Smith, 1967).

Each subject received 20 discrimination trials a day. The position of the correct gate in the brightness and the pattern discrimination task varied in a fixed manner according to a Gellerman series. If a position response was observed (defined as five consecutive responses to the same gate), that gate was blocked until the animal made a correct response (the opposite gate); after which the Gellerman series was resumed. A trial was scored as an error if a subject placed two feet in the electrified corridor front of the negative gate. The total number of errors for each subject to reach a criterion of nine out of ten correct responses was recorded.

Smith (1967) found that as infantile stimulation increased (from non-handling, handling, 0.2, 0.4, 0.6, 0.8 mA shock) the rate of learning the pattern and brightness discrimination task increased. The rate of learning as a consequence of early experience condition did not vary in a systematic manner for the position discrimination task. Thus, similar to initial set of experiments described by Levine et al. (1956), Smith (1967) provided some evidence that early infantile experience in C57BL/6 mice may affect the rate of learning, particularly as task difficulty increases.

The first study to systematically investigate the possible mediating role of temperature reduction on adult “emotionality” in an inbred strain of mouse was performed by Haggett & Werboff (1968). Notably, this study acknowledges the burgeoning work by Schaefer and colleagues that was described in the previous section on rats; and highlights some of the confounds that was present in the early work on rats:

reports by Schaefer and his associates confound body temperature reduction with thermoregulatory ability at different ages by exposure to the same environmental temperature; there is insufficient monitoring of body temperature of the rat exposed to a particular ambient temperature. Furthermore, these studies have evaluated only the rat on a limited number of behavioral parameters (Haggett & Werboff, 1968).

Two experiments were performed in the study by Haggett and Werboff (1968). The first served as a description of the “functional relationship between age and thermoregulation in infant mice and specifies the environmental temperatures necessary to provide controlled body temperature reductions throughout infancy” (Haggett & Werboff, 1968). In this experiment, 52 C57BL/6J mice from 13 litters (culled to 4 subjects per litter, 2 male and 2 female) were assigned to one of three groups. In the first

group, pups from 3 litters (6 males and 6 females) were used to establish the daily temperature of the natal nest and the daily rate of temperature loss when exposed to room temperature ($22 \pm 2^\circ\text{C}$) across PND 1-15. Using the rate of temperature loss information from these 3 litters, “approximations were made of the environmental temperatures required to produce body temperature reductions of 5 and 10°C from the average body temperature in the nest on each of the first 15 days of life” (Haggett & Werboff, 1968).

The remaining 10 litters were equally assigned (5 litters per group, 10 males and 10 females) to either a 5°C or 10°C temperature reduction condition. In these two conditions, pups were removed from the natal nest and interpolated data from the first three litters were used to determine the temperatures necessary to effect a body temperature reduction of 5 or 10°C in 10 minutes after placement in the holding compartment. Additionally, the body temperature of the pups were maintained at either 5°C or 10°C for an additional 5 minutes such that for the period between 10 and 15 minutes, body temperature and environmental temperature were equal (Haggett & Werboff, 1968).

They found that the temperature of the natal nest during PND 1-15 was relatively stable at around 34°C with no statistically significant deviations. Environmental temperatures of 26.5°C and 20.0°C on day 1, 19.0°C and 12.0°C on day 8 and 13.5°C and 10.0°C on day 15 were necessary to effect a decrease in pup temperature of 5°C and 10°C respectively.

The second study by Haggett and Werboff (1968) investigated the effects of controlled body temperature reduction (either 5°C or 10°C) of male C57BL/6J pups

during PND 1-5, PND 6-10, or PND 11-15 on subsequent adult behavior at either PND 30 or PND 70. In this experiment 75 litters were bred. Litters with less than four offspring were discarded and those with more than 8 offspring were culled. The authors stated that litters were balanced for sex but did not specify the method that was used to do so. Offspring used in this experiment were weighed on PND 1 (birth), PND 24 (weaning) and at the completion of behavioral testing (either PND 44 or 84).

Whole litters were assigned to one of five treatment conditions: 1) pups exposed to a 5°C reduction in temperature; 2) pups exposed to a 10°C reduction in temperature; 3) a non-handled control group; 4) a handled control group and; 5) a handled temperature maintained ($35 \pm 1^\circ\text{C}$) control group. There were three age groups represented within each treatment condition (PND 1-5, PND 6-10, or PND 11-15) and there were twenty subjects in each group. Of these twenty subjects per group, half were tested on PND 30 and half was tested on PND 70. Notably, this study was one of the first to systematically control for litter effects. That is, only one male and one female per litter were assigned to each behavioral test across treatment and within group.

Behavioral testing across 4 measures was conducted over a 14-day period in the following manner: 1) open field on day 1 (stress); 2) water runway on day 2 and 3 (learning); 3) water maze from day 4-12 (learning) and; 4) water submersion on day 14 (stress). The open field consisted of a gray vinyl box 36 x 36 x 18 inches in volume and marked with 6 inch squares. Latency to first square entry, number of squares entered and incidence of urination and defecation was recorded. The water runway test consisted of a gray vinyl water tank 24 x 70 x 24 inches in volume. The tank was divided into 6

runways (4 x 70 inches). Animals were placed at one end of the runway, submersed in water and latency to exit the runway (opposite end) via a ladder extending at 45° was measured. Three trials per day on 2 successive days were administered with a 5 minute break between trials.

The water maze consisted of the same water tank used in the water runway test. The first three days were spent training the subject on the task at a rate of 5 trials per day with a 5 minute interval between trials. For the remaining 7 days of testing (again 5 trials per day), subjects were tested in a single alteration spatial left-right problem (c.f., Werboff & Anderson, 1967). The latency to reach the platform, number of errors and trials to criterion were recorded. In the water submersion task, a 2 gram of tail weight to 30 gram of body weight was attached to the tail of each subject via autoclips. The subject was suspended approximately 18 inches above the water surface and then released. The time from release to 5 successive seconds of complete submersion was recorded.

No significant differences in weight were observed across treatment groups at birth (PND 1), weaning (PND 24) and at the completion of behavioral testing (either PND 44 or 84). Notably, the authors failed to specify whether a difference in weight between males and females was observed. An interaction between sex and treatment was observed when subjects were tested on PND 30 in the open field. Non-handled male subjects demonstrated the longest latencies to first square entry while non-handled female subjects demonstrated the shortest start latency to first square entry as compared to other groups. However, when subjects were tested on PND 70 in the open field, only main effects of sex and treatment were observed. Male subjects had significantly longer start

latencies than female subjects and the non-handled control group had significantly longer start latencies than the other treatment groups except for the group that was exposed to a 10°C body temperature reduction. Notably, subjects that were in the group exposed to a 10°C body temperature reduction displayed a significantly longer start latency than all of the other groups but the non-handled control group. In terms of the number of squares crossed (activity) in an open field female subjects were significantly more active than male subjects on both PND 30 and PND 70. Additionally, on PND 70 mice from the non-handled control group entered significantly fewer squares than all of the other groups except for the group exposed to a 10°C body temperature reduction. The group exposed to a 10°C body temperature reduction was not significantly different from any of the other groups in terms of the number of squares entered.

In the water runway test, only the sex of the subject and age of treatment significantly predicted the latency to escape via the runway. Females and subjects tested on PND 30 had significantly shorter escape latencies than males and those tested on PND 70 respectively.

In the water maze task the latency to reach the platform was significantly affected by treatment. Similar to the open field, the non-handled control group had a significantly shorter latency to the platform than all of the other groups except for the group exposed to a 10°C body temperature reduction. The group exposed to a 10°C body temperature reduction was not significantly different from any of the other groups in terms of their latency to the platform. Main effects of treatment, sex and age of testing were observed in terms of the number of error made. Male subjects and subjects tested on PND 70 made

significantly fewer errors than female subjects and those tested on PND 30 respectively. The non-handled control group made significantly more errors than all of the other groups and none of the other treatment groups were significantly different from one another.

Haggett and Werboff (1968) conducted separate analyses on male and female subjects in terms of time to submersion in the water submersion task. It is unclear why the decision was made to characterize sex in two separate models rather in one model as was done in all of the previous analyses. They found that regardless of sex, subjects tested on PND 30 required significantly longer times to submersion than subjects tested on PND 70. Female subjects in the non-handled control group displayed a significantly greater time to submersion than all of the other groups except for the group exposed to a 10°C body temperature reduction. The group exposed to a 10°C body temperature reduction was not significantly different from any of the other groups in terms of the time to submersion. A different relationship was observed in regards to males. The non-handled control group displayed a significantly greater time to submersion than the handled group and the group exposed to a 5°C body temperature reduction. Additionally, the temperature maintained handled group had a significantly greater time to submersion than the handled control group.

Several key findings of the study by Haggett and Werboff (1968) are worthy of re-specification. The first is that across all of the behavioral measures differences in responding were observed across males and females (generally in the opposite direction). The second is that consistent measures of behavior were generally not observed until

PND 70 (adulthood). The third is that non-handled subjects displayed more anxiety like behavior in the open-field, made more errors in the water-maze and had significantly longer latencies to submersion in the water submersion task as compared to early handled subjects. Lastly, subjects exposed to a body temperature reduction of 10°C, generally displayed a similar behavioral profile to the non-handled control group. These findings in sum do provide replication and validation of the findings that were previously described in rats for at the least, this strain of inbred mice.

The next study to investigate the effects of early handling in mice was performed by Treiman, Fulker and Levine (1970). In this study, “the temporal pattern of plasma corticosterone concentrations following electric shock was studied in 10-week old mice from two inbred strains (C57BL/10J and DBA/2) and their reciprocal crosses, half of which had been subjected to infantile stimulation” (Treiman, Fulker & Levine, 1970). It should be noted that the central theme of a gene by environment interaction on adult behavior are present in this study.

In this study two strains of mice (C57BL/10J and DBA/2) were crossed in all four possible combinations. This diallel cross produced four genetic groups: 1) C57BL/10J x C57BL/10J {C}; 2) DBA/2 x DBA/2 {D}; 3) C57BL/10J x DBA/2 {C x D} and; 4) DBA/2 x C57BL/10J {D x C}. Note, the strain of the mother is listed first in the crosses above. Litters produced for each genetic group were alternatively assigned to a handled or non-handled treatment condition at birth. Both the handled and non-handled subjects' cages were not cleaned until weaning (PND 28). The handling manipulation consisted of removing the entire litter from the cage for three minutes daily during PND 1-21. At

weaning (PND 28) subjects were sexed and housed in groups of 10 and not handled until testing (PND 70). It was unspecified whether standard husbandry procedures were maintained from weaning to testing.

The temporal pattern of the steroid response to stress was assessed on PND 70. Subjects were tested under two conditions, electric shock or control. In the shock condition, subjects were placed in a box with a grid floor and shocked twice for 30 seconds at 0.5 mA to the feet separated by 10 seconds without shock. Subjects were then placed in a holding compartment until decapitation at either 1 minute, 15 minutes or 60 minutes. Control subjects were removed from their cages and rapidly decapitated. Trunk blood was collected in heparinized tubes post decapitation and blood plasma was separated by centrifugation. Plasma corticosterone levels for each sample were determined fluorometrically.

Sixteen mice of each Sex x Genetic x Handling group were used, four at each time interval. Notably, litter effects were controlled for with one male and one female from each of two litters comprising each cell. Treiman et al. (1970) in their “full” ANOVA model found one third order interaction; Reciprocal Cross x Handling x Sex. It should be noted that the authors spend a significant portion of their paper interpreting main effects and second order interactions, which in light of their third order interactions are rendered moot with one exception; the main effect of Time. In regards to the time course of corticosterone secretion in response to stress, all subjects displayed a characteristic inverted U-form, with a peak in corticosterone secretion being observed at 15 minutes and a decrease observed by 60 minutes.

In terms of the third order interaction; Reciprocal Cross x Handling x Sex several important findings emerge. The first is that in both handled females and handled males no significant differences were attributable to the genetic background or maternal environment. Secondly, non-handled reciprocal crossed males do not differ in terms of their corticosterone secretion response to stress from their maternal strain. Conversely, non-handled females differed significantly from their maternal strain in regards to their corticosterone secretion response to stress. Non-handled DBA/2 females displayed a significantly elevated response curve as compared to DBA/2 x C57BL/10J crossed females but are not significantly different from the C57BL/10J x DBA/2 crossed females. Conversely, non-handled C57BL/10J females displayed a significantly lower response curve as compared to C57BL/10J x DBA/2 crossed females and a significantly higher response curve from the DBA/2 x C57BL/10J crossed females. Thus, for non-handled females, the pattern of corticosterone secretions in response to a stressor was found to be contingent on the strain of the mother.

The results of the study by Treiman et al. (1970) are interesting in a number of regards. First, it replicates previous findings in rats which specify that corticosterone secretions in response to a stressor can be modified by early handling. Secondly, it specifies that the patterns of corticosterone secretions in response to a stressor may vary as a function of the strain of the mouse. It cannot be concluded from this study whether this observed difference is a function of the genetic background of the mouse, a function of the maternal milieu experienced by the pup or a combination of both.

The next study to investigate the effects of early handling in mice was performed by Bell et al. (1971). By this time, the temperature hypothesis was gaining some support, but results varied across laboratories. Bell et al. (1971), noting that when mice were placed in an unheated environment a concurrent increase in USVs production was observed, hypothesized that handled mice would vocalize more when returned to the natal nest as compared to non-handled controls.

This study utilized four litters of deermice. Bell et al. (1971) stated that "this species was selected because their ultrasonic signals fall within the frequency limitations imposed by the available recording equipment." Handling occurred during PND 1-10 and consisted of moving the entire cage to an area outside a sound recording room for twenty minutes daily. Thereafter, individual pups were removed from the litter and placed in a small chamber for 3 minutes, and then returned to the home cage. On days, 1, 2, 3, 5, 7, and 10 the cage was placed in the sound room post reunion with the dam, and sound recordings made 0-30 and 180-210 seconds after the cage was positioned under the recording microphone. The non-handled comparison group was treated identically, with the exception that the pups were never removed from the cage. The authors found that early handled subjects signaled more often, and also signaled at higher peak frequencies with longer mean durations than non-handled subjects. Thus, in the 1970's, the production of USVs were demonstrated to be an important discriminatory measure when evaluating the effects of handling in mice.

In 1972, Porter investigated the effects of the "environmental manipulation" of early handling on the of the inter-strain dominance hierarchy (a consequence of what he

terms “inborn behavioral dispositions”) between male C57BL/10 and BALB/c inbred mice. Note that the underlying theme of separation of gene from environment is present in this study. Pups from an unspecified number of C57BL/10 and BALB/c litters were either assigned to an early handled or non-handled group (12 / group / strain). Handling consisted of placing each dam in a holding cage and each pup into its own cardboard container for three minutes daily during PND 2-16. The non-handled groups were undisturbed until weaning and all of the groups were weaned on PND 25. Food and water were provided *ad libitum*. Subjects were housed in same sex and litter cages and only male offspring were tested as adults.

On PND 50 all subjects were isolation housed for 10 days until the beginning of testing for dominance on (PND 60). Assessment of dominance within a group and across strain consisted of:

 pitting handled and non-handled C57BL/10 males against handled and non-handled BALB/c males in a 2×2 design. Each series of such inter-strain encounters between BALB/c vs. C57BL/10 mice consisted of a round-robin order of fights with 12 mice from each strain in each round-robin series. Thus, in each of the 4 test series, 12 BALB/c mice (either handled or non-handled) were paired one at a time against each of 12 C57BL/10 mice so that each subject engaged in 1 inter-strain encounter/day for 12 consecutive days. Each of the 12 mice of a given strain-condition combination was pitted against each of the 12 mice of the opposite strain and of only 1 of the 2 experimental conditions (H or NH). The order of paired encounters within a round-robin series was randomly determined prior to the initial encounter (Porter, 1972).

In each encounter two subjects were placed at opposite corners of a two compartment chamber and isolated for 2 minutes. After two minutes the partition that isolated the subjects were removed and the patterns of interactions between the two

subjects were observed. Aggression was operationally defined as “as actual physical contact involving biting-either mutual biting or one animal attacking and biting the second while dominance was operationally defined as “occurring when one animal repeatedly attacked the other with impunity, while the subordinate animal adopted a submissive posture (e.g., standing on its hind legs with its forepaws extended) or fled from its attacker” (Porter, 1972). Each subject was assigned a dominance score with one point awarded for a dominant encounter and one negative point awarded for a subordinate one.

Porter (1972) found that regardless of handling condition C57BL/10 were dominant to BALB/c mice. Handled BALB/c mice were found to be more submissive to C57BL/10 mice (regardless of handling condition) than non-handled BALB/c mice. This study was the first to describe the differential manifestation of phenotypic outcome in inbred mice strains as a consequence of handling. However, these results should be interpreted with caution as the effects of handling are confounded with social isolation previous to testing. In addition, this data is heavily confounded because for each mouse the outcome of every encounter may have had an effect on subsequent encounters on (except for the first encounter).

Hucklebridge and Nowell (1973) were the first investigators to measure the effects of early handling in mice on the secretion of the catecholamines epinephrine and norepinephrine from the adrenal medulla in response to stressor as an adult. In experiment 1 of this study, and 12 pairs of tuck albino T.O. mice were mated at 9 weeks of age with food and water *ad libitum*. Regular husbandry was suspended during breeding

and gestation with only one cage change occurring. The litters derived for this experiment were born on either 20, 21 and 22 days after mating. On the day of birth, litters were sexed and culled to six pups (including at least four males); stud males were removed and litters were randomly assigned to either a handled ($n = 6$ litters) or non-handled condition ($n = 6$ litters).

Handling occurred during PND 1-20 and consisted of “removing the pups from their mothers, gently rolling them in the palm of the hand and placing them individually into small cages ($30 \times 12 \times 12$ cm) lined with fresh sawdust” (Hucklebridge & Nowell, 1973). Subjects were returned to their home cage after 5 minutes. Litters were weaned, weighed, and re-sexed on PND 21. The authors stated that “four males were randomly selected from each litter and housed together in Makrolon cages ($28 \times 21 \times 10$ cm) (four littermates per cage), supplied with food and water ad lib” (Hucklebridge & Nowell, 1973). However, given that at birth litters were culled to 6 subjects, of which, at least four were males, one might question the purported “randomness” of this cage assignment.

On PND 60, mice from both the handled or non-handled treatment conditions were assigned to one of three adult “stressor” conditions: 1) a non-shocked control; 2) shocked followed by immediate blood sampling and; 3) shocked followed by 15 minutes of recovery before sampling. In total there were six treatment categories with 10 subjects per treatment. The remaining 12 subjects were used as “extras” in the event that blood sampling was unsuccessful. The shock procedure consisted of placement of the subject into a Perspex box for 5 minutes, where 1.0 mA of shock was delivered to the feet of the

subject for 30 seconds followed by 30 seconds of no-shock. Thus, subjects in the shock condition received five 30 second intervals of shock.

Immediately after exposure to the stressor, subjects were transferred to an anesthetizing jar where they were anesthetized with ether. The subject's body cavity was then opened and approximately 1 milliliter of blood was obtained from the inferior vena cava (between the diaphragm and the right auricle) using a heparinized needle. Blood samples were taken at 5 minutes post exposure to anesthesia. The authors acknowledged that while the stress involved in sampling blood from an anesthetized subject may result in massive secretion of catecholamines from the adrenal medulla, a pilot study in their laboratory indicated that there was no elevation in plasma catecholamines between 2.5 minutes and twenty minutes post exposure to anesthesia.

Blood samples were then transferred to ice-cooled centrifuged tubes and centrifuged at 3000 rpm at 0°C for ten minutes to separate plasma. Plasma was then transferred to plastic vials stored at 0°C and then assayed on the same day for epinephrine and norepinephrine. The authors stated that the assay used was sensitive to nanogram quantities of catecholamines.

Hucklebridge and Nowell (1973) found that for both handled and non-handled subjects exposure to an electric shock stressor led to a 2-fold increase in plasma epinephrine. Similarly, plasma epinephrine levels returned to the similar levels as the controls in both the handled and non-handled groups respectively after 15 minutes post onset of stressor. Lastly, plasma epinephrine levels between handled and non-handled subjects were not found to be different across any of the adult treatment groups (i.e., non-

shocked, shocked with blood taken immediately, shocked with blood taken after 15 minutes). Conversely, while plasma norepinephrine levels were significantly elevated in both shocked handled and shocked non-handled subjects, this elevation was significantly greater in shocked handled mice. Plasma norepinephrine levels did not differ between non-shocked handled and non-shocked non-handled mice and in both handled and non-handled mice, plasma norepinephrine levels had returned to values similar to their respective non-shocked controls after 15 minutes. Thus, handled mice seem to exhibit greater secretions of norepinephrine after exposure to an electrical shock stressor. The fact that no differences in plasma norepinephrine were observed 15 minutes after exposure to a stressor, only suggests that handled subjects mount a more rapid response to a stressor. It is unclear given the present data whether a quicker but shorter response (in the form of secretion of hormones from the adrenal medulla) also occurs in handled subjects.

In experiment 2, Hucklebridge and Nowell (1973) investigated whether exposure to a social rather than an electrical stressor elicited different adrenal activity in handled and non-handled subjects. The methodology and numbers of subjects employed in experiment 2 was reported to be identical to that of experiment 1 (at least until testing as an adult). Additionally, in experiment 2 the authors removed the adrenals of their subjects as an entire section in their paper is devoted to “Assay of Adrenal Epinephrine”. However, no description of the procedure during which the removal of the adrenals occurred can be found in this paper. However, in order to evaluate the efficacy of their assay on the adrenals, details on the methodology as well as the time frame in which the

adrenals were removed and frozen are necessary. Regardless, it was stated that adrenals were homogenized, centrifuged, the supernatant fluid pipette off and assayed for levels of only epinephrine. The authors stated that only epinephrine was assayed as this “technique is not suitable for differentiation between epinephrine and norepinephrine when the proportion of norepinephrine is small as in the adrenal medulla” (Hucklebridge & Nowell, 1973).

On PND 60, subjects were exposed to a social stressor, operationally defined by the authors as defeat by a trained fighter mouse. The authors stated that as no differences between handled and non-handled subjects was observed after 15 minutes in experiment 1 (in terms of plasma epinephrine and norepinephrine); the time course of catecholamine response post exposure to a stressor was not investigated. Subjects were therefore assigned to one of two conditions: 1) placement in the cage of a fighter mouse without actual physical exposure to the fighter mouse (control) or 2) defeat by a fighter mouse. As in experiment 1, subjects exposed to a stressor were immediately anesthetized and blood sampled within 5 minutes.

In this paradigm, an experimental mouse is introduced into the home cage of a socially isolated trained fighter mouse and separated from the fighter mouse by a wire mesh. After 1 minute the mesh is removed and the fighter mouse “attacks the intruder.” The period of exposure to the fighter mouse was five minutes and only subjects which submitted to the fighter mouse (i.e., were attacked for less than 50 seconds or received fewer than 40 bites) were considered in the author’s analysis. These exclusionary criteria according to the authors were used so that “some form of control was exercised over the

intensity of social stress” (Hucklebridge & Nowell, 1973). In addition, “comparison between different treatment categories subjected to this form of stress was considered meaningful only if there was no significant difference in the intensity of stimulation as judged by these two criteria” (Hucklebridge & Nowell, 1973).

Hucklebridge and Nowell (1973) found that while both handled and non-handled groups had a significant increase in the elevation of plasma epinephrine secretion in response to a “social stressor”, this elevation was significantly greater in handled subjects. Plasma norepinephrine levels were also significantly elevated in both handled and non-handled groups in response to a “social stressor”. However, this elevation was not significantly different between handled and non-handled subjects. No significant differences were observed between handled and non-handled subjects in either the defeat or control condition in terms of adrenal weight, and adrenal epinephrine concentration. Thus, in this study, handled subjects showed a quicker response to an electrical stressor (in the form of elevated norepinephrine levels) and to a social stressor (in the form of elevated epinephrine levels) as compared to non-handled subjects.

At this point in history, there was increasing support for the maternal mediation hypothesis in explaining the effects of early handling on adult behavioral development. Priestnall (1973) was the first to attempt to systematically describe the effects of handling of either the mother (maternal handling) or the pups (early handling) on subsequent maternal behavior (i.e., behavior performed by the mother) in mice. In this study, 30 pregnant multiparous CFLP mice were mated and checked daily for pups. At parturition litters were culled to ten pups. Subjects were maintained on a reverse 12-12 light dark

cycle and observation of maternal behavior in the dark phase was facilitated by four 100 watt red coated bulbs.

Seven days after birth (i.e., PND 7) whole litters were randomly assigned to a maternal handled (MH), early handled (EH) or non-handled (NH) control group. The early handling procedure consisted of removal of the entire litter from the nest and placement in a can partially filled with wood shavings for four minutes. Maternal handling consisted of exactly the same procedure as early handling where the mother was removed rather than the pups. Both handling procedures occurred during PND 7-10 at an unspecified time.

Priestnall (1973) stated that for the non-handled group “neither the litters nor the lactating females were handled in this group.” No description of the husbandry procedure was provided in this study, and it remains unclear whether any of the treatment groups and more importantly the non-handled group, was manipulated for weekly husbandry procedures. This distinction is important because research in rats it has suggested that subjects that experience standard animal husbandry procedures (Animal Facility Reared, AFR) look similar in terms of their behavioral development to Early Handled subjects.

Observation of maternal behavior for all groups began immediately after subjects were returned to their cages. It was stated that “the experimenter moved from cage to cage, noting the behaviour of each female on a prepared protocol. The record was made in coded form using a code developed by Priestnall (1970)” (Priestnall, 1973). [I was unable to retrieve a copy of the Priestnall (1970) dissertation and I therefore am unable to elaborate in detail on this coding protocol.] From the Priestnall (1973) paper it is

understood that there were 60 observation periods which spanned a period of four hours subsequent to the reuniting of the pups and the dams in the home cage. Each observation period encompassed a 4 minute interval and frequencies of maternal behavior were recorded. It is unclear what criteria were used in establishing the frequency of behavior. Given that the experimenter moved from cage to cage noting maternal behavior it may be assumed that for dichotomous scoring was used (i.e., present or absent) for a given observation period.

Summary scores of behavior for each hour on all four days of testing per litter were calculated and submitted to an ANOVA. Behaviors of low frequency which violated the assumptions of parametric statistical analysis were submitted to a Kruskal-Wallis non-parametric ANOVA. The following behaviors were assessed: 1) out of nest; 2) inside of nest; 3) active but not maternal; 4) still but not maternal; 5) grooming self; 6) eating 7) arch backed nursing; 8) licking offspring and; 9) nest-building.

Priestnall (1973) found that dams in the early handled treatment condition licked pups significantly more than the mother handled and non-handled groups. Furthermore, this difference in licking behavior was manifest 1 hour after reuniting with the pups. The only other difference in maternal behavior that was observed was found in regards to grooming. Dams in the early handled treatment condition groomed themselves significantly more than the mother handled and non-handled groups. This study is important because it specifies that in CFLP mice, the consequential up-regulation of maternal behavior (in this case, licking) is a direct consequence of removal of the pups from the natal nest rather than the mother. It is unclear which variable or combination of

variables specifically mediates this increase in maternal licking behavior observed in early handled litters, although temperature reduction of the pups as well as the corresponding change in the stimulus properties of the pups are implicated.

Sherrod, Connor and Meier (1974) were one of the first sets of investigators to report on time course of the increase in maternal responsiveness as a consequence of early handling. Specifically, the authors were interested in: 1) whether quantitative or qualitative differences exist in mother-infant interactions between handled or control litters; 2) whether these differences in mother-infant interactions persist until weaning or exist only immediately after handling; and 3) whether cross-fostering has an influence on the effects observed due to handling.

In this study 12 BALB/c primiparous dams and their litters were used as subjects. Subjects were housed in 23 x 18 x 13 cm clear plastic cages with alfalfa bedding, and standard animal husbandry procedures occurred weekly. Subjects were maintained on a 14/10 light dark and the ambient sound level were around 60dB.

Litters were designated as either handled or non-handled, in alternating order, on PND 1 (day of birth). Handling consisted of removing each pup individually from the cage and placement onto a table top for 5 minutes. Subjects were handled or non-handled during PND 1-7 and then either reared by their biological mother, or cross-fostered to a dam that had reared either handled or non-handled pups during PND 1-7 until weaning on PND 21. Two litters were therefore assigned to each of the following 6 conditions: 1) early handling during PND 1-7, not cross fostered; 2) non-handled during PND 1-7, not cross-fostered; 3) non-handled during PND 1-7, cross-fostered to a dam from a non-

handled group; 4) handled during PND 1-7, cross-fostered to a dam from a non-handled group; 5) non-handled during PND 1-7, cross-fostered to a dam from a handled group; 6) handled during PND 1-7, cross-fostered to a dam from a handled group.

For each dam, 5-minute observations in 5 second epochs occurred once daily on mother and infant behavior. Note, during PND 1-7 observation of handled litters occurred twice, pre-handling and post-handling. Dams were scored every 5 seconds on the following behaviors: 1) licking or manipulating pups; 2) retrieving pups to nest; 3) cleaning or digging at the nest; 4) nursing; 5) carrying pups (not toward nest); 6) retrieving own tail; 7) grooming self; 8) out of nest and; 9) eating. These behaviors were mutually exclusive and there was no observation epoch in which the dam was not found to be engaging in one of these activities. Of note, the authors stated that “if an animal was scored as engaging in one behavior, it could not be simultaneously scored for another.” Thus for a given epoch, only one behavior was scored and is approximated as occurring for 5 seconds. Infants were scored on whether there were in the nest or eating from the hopper.

They found that during PND 1-7 dams of handled pups spent more significantly more time stimulating the pups upon reunion. That is, time spent licking, carrying, and retrieving pups, cleaning the nest and out of the nest all increased upon reunion with the dam. Time spent performing maternal behavior(s) prior to the handling manipulation was not significantly different from the non-handled group. Thus, if changes in maternal behavior are observed as a consequence of early handling, these changes seem only to be

manifest during the post-reunion period. It should be noted that change across PND is not described in this analysis even though the pre- and post-reunion data is nested within it.

Only the behaviors of time spent licking, eating, self-grooming, out of nest and nursing were analyzed during PND 8-21. In this analysis an interaction between cross-fostering and handling condition was observed. Pups that were either handled during PND 1-7 and then cross-fostered to a dam that previously reared non-handled pups or non-handled during PND 1-7 then cross-fostered to a dam that previously reared handled pups were licked more than pups that were non-handled during PND 1-7 and then reared cross-fostered to a dam who previously reared non-handled pups and handled pups reared by a dam who cross-fostered to a dam who previously reared handled pups. In other words, if the dam reared offspring from the treatment group during PND 8-21 that was different to what they reared during PND 1-7, licking behavior increased. This increase in licking behavior may be a consequence of the discordance in the patterns of interactions between the pup and the dam.

Recall, infant specific behaviors of time spent out of nest and eating from food hopper were also recorded. The authors reported that these behaviors were low in frequency until late in the pre-weaning period and thus data for infants were analyzed only for the last eight days of the pre-weaning period (PND 14-21). Handling did not differentiate between groups of infants but the frequency of these behaviors increased during PND 14-21.

At this point in history, the hypothesis that an increase in licking behavior as a consequence of early handling mediates adult behavioral development in both rats and

mice was gaining support and was largely favored. Thus, various researchers began to investigate whether a general increase in tactile stimulation provided to the pups rather than an increase in licking behavior itself could “produce” offspring that displayed a “blunted adult emotional” profile that was characteristic of early handled subjects.

Recall, application of the early handling treatment generally occurs during PND 1-10. The study by Sherrod et al. (1974) specified that handling during PND 1-7 increases levels of maternal responsiveness (licking, carrying, and retrieving pups) of the dam to the pups upon reunion. LaBarba, Fernandez, White and Stewart (1974) in a complement to the study by Sherrod et al. (1974) were among the first to describe the effects of increases in tactile stimulation of offspring behavioral development in mice.

In the study, eighteen litters of BALB/c “were randomly selected from a large number of litters of 5 or more pups” (LaBarba et al., 1974). Litters were culled to 5 pups within 24 hours after birth. All litters were derived from nulliparous females. In total 90 subjects were used, 49 males and 41 females. Subjects were maintained at a stable temperature of 27°C.

The eighteen litters were randomly assigned to the following 9 treatment groups (2 litters of 5 pups each): 1) subjects received two minutes of tactile stimulation in an incubator; 2) subjects received two minutes of tactile stimulation at room temperature; 3) subjects received five minutes of tactile stimulation in an incubator; 4) subjects received five minutes of tactile stimulation at room temperature; 5) a control group that was placed in the incubator for two minutes; 6) a control group that was exposed to ambient room temperature for two minutes; 7) a control group that was placed in the incubator for five

minutes; 8) a control group that was exposed to ambient room temperature for two minutes and; 9) a non-handled control group that was undisturbed until weaning. Note, each experimental group (i.e., ones that received tactile stimulation) had an associated yoked control.

All treatments occurred during PND 1-10. Tactile stimulation to the pups was administered using a 36 centimeter sable hair brush. First, a litter from an experimental group and a litter from the experimental group's yoked control were placed in an incubator at 27°C. The dams were then removed from the home cage and placed in identical holding cages. Each pup in the experimental group in question was then brushed in a cephalocaudal direction at a rate of 58-60 strokes per minute for the pre-specified time. At the end of each treatment session, the dams were replaced in their home-cages. Subjects were weaned on PND 21 and isolation housed for the remainder of this study.

On PND 50 subjects were tested on a daily 3 minute trial for "emotionality" in a quadrant activity cage across 3 consecutive days. Data for males and females were collected separately was analyzed in a 2 x 2 x 2 x 2 factorial design using an unweighted means solution for ANOVA. They found that subjects that received two minutes of tactile stimulation were more active, transversed more quadrants and had lower defecation rates (in sum, less "emotional") than subjects that received 5 minutes of tactile stimulation, the 2 minute and 5 minute yoked controlled groups and the non-handled control groups. Conversely, subjects that received 5 minutes of tactile stimulation were not found to be different from the non-handled controls in terms total activity and number of quadrants crossed. Interestingly, the 2 minute and 5 minute yoked controlled groups were more

active, transversed more quadrants and had lower defecation rates (in sum, less “emotional”) than subjects that received 5 minutes of tactile stimulation. A follow-up ANOVA employing a hierarchical design did not support the notion that the effects observed in this study was a consequence of having subjects in a litter nested within treatment. Thus, the data from this study suggests that “the duration or intensity of the tactile stimulation determines the direction of the effect” (LaBarba et al., 1974).

Watson, Henry and Haltmeyer (1974) were the first to describe the additive effects of “infantile stimulation and postweaning socialization make to CBA mice in subsequent emotional reactivity and response to a complex social environment”. In this study, forty litters of CBA mice were bred, with half of the litters randomly assigned to the early handled condition (n=20) and the other half assigned to a non-handled comparison group (n=20). The handling manipulation occurred during PND 1-20 and consisted of removing the pups individually and placing them on shavings for 3 minutes with no active attempt to regulate the temperature of the pups. On PND 21, half of the males and half of the females from each litter were randomly assigned to isolation or social housing. Subjects were isolation housed in a “quart jar” wrapped in a paper towel (to prevent visual contact with other animals) and were provided with food and water *ad libitum*. Subjects were socially housed with 7-10 animals in a 17×28×13cm “nest box” and were also provided with food and water *ad libitum*.

On PND 40, subjects were evaluated for “emotional reactivity” in an open field across four days of testing. Seven females and eight males were randomly selected from each group (i.e., early handled-post-weaning socialized, early handled-post-weaning

isolated, early non-handled-post-weaning socialized, and early non-handled-post-weaning isolated) to be decapitated, and have their trunk blood collected and assayed for plasma levels of corticosterone. Subjects in the handled-socialized group displayed the greatest locomotor activity in the open field across four days of testing. No habituation response was observed in the open field, as levels of locomotor activity across all groups were not statistically different across the four days of testing. A corresponding physiological effect was also observed, with early handled-post-weaning isolated, early non-handled-post-weaning socialized, and early non-handled-post-weaning isolated subjects displaying significantly higher levels of plasma corticosterone as compared to early handled-post-weaning socialized subjects. While direct comparison to other studies is not possible here, the results of this study suggest that early handling followed by social housing (as is commonly found in standard animal husbandry procedures) attenuates behavioral and physiological responses to a novel and open environment.

There is a noticeable gap of about 6 years in the literature describing the effects of or investigation into the mechanisms behind early handling *in mice*. The next study in a *PubMed*TM search was performed by Hennessy, Li, Lowe and Levine (1980). In this study, changes in maternal behavior as a consequence of handling in two strains of inbred mice (C57BL/6 and A/J) were investigated. Of note, the central theme of a gene by environment interaction on adult behavior is present in this study as it was stated: “We used a cross-fostering design to distinguish those strain differences due to the genotype of the mother from those due to the genotype of the pup” (Hennessy et al., 1980).

Virgin female adult C57BL/6J and A/J mice maintained on a 12/12 light dark cycle with food and water *ad libitum*, were bred with males of their respective strain. Females were transferred to individual cages about 4 days before parturition. On the day of birth (PND 1), dams were given nesting material and pups were redistributed to dams to create the following four groups: 1) C57BL/6J dams given C57BL/6J foster pups; 2) C57BL/6J dams given A/J foster pups; 3) A/J dams given C57BL/6J foster pups and; 4) A/J dams given A/J foster pups. Foster litters contained between 4 and 6 pups and any mother that did not maintain at least four pups until weaning were discarded.

Handling occurred daily, but observations of maternal behavior were made pre- and post-handling between 1200 and 1700 hours only on PND 3, 5, 6, 9, and 11. For the pre-handling observations, the experimenter looked into each cage once every 2 minutes and noted any occurrence of the following dam behaviors: out of nest, nursing, licking, nest-building, self-grooming, rearing, feeding and drinking. Time spent out of nest by the pups was also recorded. A total of 15 observations per cage were made pre-handling. Following the pre-handling observations, each cage was transferred to an adjacent room. The dam was then removed from the cage and placed in a holding cage. The pups were then removed individually and placed in a 500ml glass jar with wooden chips for two minutes. The pups were then returned to the edge of the home cage farthest from the nest and the dam was then replaced into the home cage. A cage top with neither food nor water was then placed on top of the cage. This permitted for uninterrupted observation of maternal behavior post-handling for the next 20 minutes.

Post-handling scoring of maternal behaviors consisted of the latency to retrieval of the first pup. If the dam built a nest around the pups rather than retrieving them to the nest, the latency from the start of the observation to the time when the mother placed the first piece of nesting material around the pups was scored. Additionally, observations of nursing, licking, in nest with all pups, nest-building, self-grooming and pup-carrying were made every 30 seconds for the 20 minute observation session. At the end of the 20 minute observation, the percent of pups retrieved to the nest was recorded.

The measures of nursing pre-handling and retrieval post-handling were submitted to separate 3-way (strain of mother x strain of pup x days) ANOVAs, with day treated as a repeated measure. Other dependent measures (pre-handling: out of nest, self-grooming, feeding; and post-handling: nursing, nursing latency, nest-building, pup-carrying and self-grooming) were summed across the five days of observations (as they were low in frequency) and submitted to a 2-way ANOVA. For 3 additional measures (pre-handling: pup-licking, nest-building, and pup-licking), the summing of behavior across days of observations was insufficient to permit for the use of standard parametric statistical analyses, therefore Mann-Whitney U tests were used to evaluate significance. The pre-handling measures of rearing, drinking and pups out of nest were observed rarely (i.e., not observed for at least half of the dams) and thus were eliminated from the analysis. Square-root transformations were used to normalize the pre-handling nursing and the post-handling retrieval latency and nursing latency distributions.

Pre-handling, a significant strain of mother x strain of pup interaction was observed for nursing, out of nest and self-grooming. Dams were observed to nurse more,

be out of nest more and self-groom less if they were caring for their own pups than pups of the other strain. A main effect of day was also observed for nursing. Dams spent more time nursing during the earlier days of lactation than in the later days. No other differences were noted pre-handling.

Post-handling, C57BL/6J dams were found to have a shorter latency to retrieve pups, nest build more, and nurse, carry pups and self-groom less than A/J mothers; regardless of the strain of the pup being cared for. Dams of both strains were found to initiate retrieval faster if they had A/J foster pups but were observed to lick C57BL/6 foster pups more. No other effects were observed.

The results of this study highlight the possible mediating effects of strain differences in maternal behavior and in pup ability to elicit maternal behavior as a consequence of handling. However, “although considerable phenotypic variation in individual components of maternal behavior was observed, no clear-cut strain differences appeared in the overall quality of maternal care or in the overall capacity of pups to elicit maternal care” (Hennessey et al., 1980). In other words, even though it could be suggested that the C57BL/6J dams were better at expressing maternal care because they were quicker at retrieval and engaged more frequently in nest building as a consequence of handling, the same could be argued for A/J dams as they engaged in more frequent nursing post-handling. It is unclear in this study whether within strains, handled subjects were significantly different in patterns of maternal responsiveness from non-handled subjects or whether the pups of one strain were better at adjusting their behaviors as a consequence of handling because no such comparison (control) group was used.

In a second experiment, Hennessy et al. (1980) investigated the hypothesis that increased production of USVs as a consequence of handling may have led to the more rapid retrieval of A/J pups than C57BL/6J pups in the first experiment. Additionally, this experiment examined the relationship between pups' USV signaling and regulation of body temperature as "strain differences in thermoregulatory capacity could potentially underlie any strain difference which might be observed in ultrasonic signaling" (Hennessy et al., 1980).

Specifications for the subjects, housing and fostering conditions were identical to that of the first experiment. An USV detector equipped with a pre-amplified microphone and an adjustable frequency tuner were used to assess ultrasonic signaling. These signals were processed with a solid state conditioner and output was recorded with a counter. The conditioner determined the presence or absence of USVs during successive intervals of 75ms. Pilot testing indicated that a frequency of 68 kHz was optimal for detection of USVs from pups of both strains in this age range. During testing, the microphone was positioned 12cm above the pups on a ring stand. Axillary rather than rectal body temperature was recorded as it constituted a less invasive and less stressful procedure and was determined using a microprobe thermometer.

Handling occurred daily but subjects were monitored only on PND 3, 5, 7, and 9 for USV production and changes in body temperature. Axillary body temperature of a single randomly selected pup was recorded prior to and subsequently after handling. After replacement of the pups in the home cage ultrasonic vocalizations were monitored for 4 minutes (as nearly all dams had begun retrieving pups by 4 minutes in experiment

1). Note, the dam remained in her holding cage during this period. After the 4 minutes had passed, the dam was replaced into the home cage. The home cage was then left undisturbed for 15 minutes at which time the auxiliary body temperature of one randomly selected pup was recorded.

C57BL/6J mice were found to signal only occasionally in the four minute interval subsequent to handling. In 20 of the 40 sessions in which C57BL/6J mice were tested no USVs were detected. Conversely, A/J mice consistently produced USVs on each test day. This difference in the production of USVs between C57BL/6J and A/J mice were found to be statistically significant. The strain of the rearing dam did not impact the production of USVs. All A/J litters showed a reduction in USV signaling upon reunion with the dam (regardless of strain) whereas no clear pattern of USV signaling pre- and post-reunion was observed in C57BL/6J mice. In addition, C57BL/6J pups decreased in temperature at a significantly slower rate when removed from the natal nest and displayed a more rapid increase in temperature upon reunion with the dam as compared to A/J mice. Together, these experiments suggest that it may be important to understand strain differences in pup and maternal behaviors and endogenous temperature regulation related to early handling as these may mediate the types of “emotional” phenotypes observed as a consequence of early handling.

Thus, initial studies of the consequential effect of early handling in mice demonstrated an up-regulation of maternal behavior similar to that which was identified in rats. While it is outside the scope of this paper to detail this line of research, it is worth noting that those studies which demonstrated that the strain of the mouse may have an

interactive effect with the application of the early-handling treatment may have served to re-orient lines of research toward investigation into the effects of cross-fostering inbred mice to unlike inbred dams and quantification of the concordant changes in maternal behavior (e.g., Ward, 1980; Carlier, Roubertoux, & Cohen-Salmon, 1983, Zaharia et al., 1996).

One additional study is worthy of mention. Although the literature in rats had at this point in history demonstrated some positive effects of handling on immune responsivity, similar work had not been replicated in mice. Lown and Dutka (1987) were one of the first groups to investigate the effects of early handling on immune function development in mice. “One objective of these experiments was to examine the effect of early handling on both the humoral and the cellular components of the immune response using mitogen stimulation of splenic B and T cells” (Lown & Dutka, 1987). Additionally, a second experiment assessed whether the effects of early handling were limited to the pre-weaning period. Therefore, subjects in this study were handled either pre-weaning or post-weaning. In my review of the literature, this seems to be the first study to validate the critical period hypothesis in mice (recall, Levine & Otis (1958) were to first to do this in rats).

Six newborn C3h/St litters of mice were culled to four pups (two males and two females) within 12 hours of birth. Litters were randomly assigned to either a handled or non-handled group with a total of twelve pups (3 litters, 6 males and 6 females) per condition. Subjects were housed in plastic maternity cages with sawdust bedding in a

17/7 light dark cycle. Temperature was maintained at 25°C and food and water were provided *ad libitum*.

The handling procedure consisted of removal of the dam from the home cage and placement into a holding cage. Each pup was then removed individually and placed in a small glass jar filled with sawdust bedding for 3 minutes. The pups and then the mother were returned to the home cage. Non-handled litters were not disturbed until weaning. Handling occurred between 1400 and 1530 hours daily from birth until weaning on PND 21. On PND 21, subjects were sacrificed, their spleens were removed and mitogen assays were performed. This assay provided mean counts per minute of mitogen induced B- and T-cell proliferation. No significant effects of handling on B- and T- cell proliferation were found.

In a second experiment, Lown and Dutka (1987) investigated whether handling pre- vs. post-weaning may have an effect on immune function development. Specifications for subject housing and manipulation pre-weaning were identical to that of the first experiment. On PND 21, subjects in the handled and non-handled conditions pre-weaning were subdivided into post-weaning handled and non-handled groups. Thus, there were four groups in total, with an average group size of 9 to 11 subjects. It was unspecified how many litters constituted the subjects for this experiment and whether the number of males and females were balanced across groups. Given the variation that was present in the group size, it is tentatively assumed that litter size and sex were not balanced across groups. Post-weaning handling consisted of removal of subjects from their home cage and placement on top of the metal top of a plastic maternity cage where

subjects were allowed to explore freely for 10 minutes. Post-weaning handling occurred between PND 21-60. On PND 60 subjects were sacrificed, their spleens were removed and mitogen assays were performed.

A 2 (pre-weaning handling) x 2 (post-weaning handling) between subjects ANOVA revealed a significant main effect of pre-weaning handling for both B- and T-cell proliferation. That is, subjects that were handled pre-weaning had significantly higher B- and T- cell proliferation responses. Thus, the results of this experiment suggested that early-handling does have an effect on the development of immune function and that these differences are manifest only later in adulthood. This experiment also demonstrated that similar to rats, post-weaning handling does not have a statistically significant effect on the development of immune responses.

- ***Summary of the Effects of Early Handling in Mice***

In summary, the literature suggests that the effects of EH when compared to a NH group are robust as in the research in rats and appear to be under multiple sensory and redundant control *within strains of mice*. More specifically, the studies outlined in the preceding chapter do suggest that the causal pathway via which a blunted “emotional” profile emerges in offspring as adults does share some homology with the work that has been previously described in rats. Importantly, strain differences in patterns of maternal care may mediate the relationship between early handling and subsequent adult “emotionality”. Therefore, studies investigating the role of early handling on later “emotionality” should make some attempt to systematically control for strain differences

in maternal care when assessing the effects of early handling across strains. Other variables of consider include but are not limited to, pup temperature regulation post separation and differential production of USVs post reunion across sexes because both of these affect maternal care. Also, studies should control for differential patterns of maternal care exhibited to male and female offspring, litter effects, and the use of appropriate statistical analyses.

Maternal Separation in Rats and Mice

While it is outside the scope of this paper to review in full the range of experimental treatments that fall under the paradigm of maternal separation (MS), an overview of the most widely used paradigms that fall under this umbrella term of maternal separation is necessary for effective evaluation of the EH model. In the 1970s, the paradigm of maternal separation was introduced as a consequence of Bowlby's (1969) hypothesis that separation of the mother from the infant early in development may be responsible in part for adult later social and emotional problems. This research was originally formulated in primates but has also been used extensively with rodents (e.g., Hofer, 1987).

In the rodent literature, the term maternal separation (MS) or maternal deprivation (MD) is used collectively to describe a variety of manipulations which involve separation of the dam from the pups for different time periods (single or repeated separations for different time periods, 1-24 hours). The three most common forms of maternal separation are: Maternal Separation (MS) which involves removal of the dam from the littermates

for three to six hours (depending on the specific study) during PND 2-14; Single Maternal Separation (SMS) which involves separation of the dam from the littermates *once* for 8 to 24 hours (depending on the specific study) sometime between PND 2-14 (again, dependent on the study) and Maternal Peer Separation (MPS) / Early Deprivation (ED) which involves separation of the dam from the littermates, and the littermates from one another, for three to six hours (depending on the specific study) during PND 2-14. However, procedures as diverse as artificial rearing between birth and weaning, and daily separation for up to 16 hours, have all been reported in the literature under the umbrella term of maternal separation (Lehmann & Feldon, 2000).

At present, research into the effects of maternal separation in rodents is confusing at best. There is some evidence indicating that maternal separation (MS) produces effects that are opposite to EH (e.g., Huot et al., 2001). Huot et al. (2001) compared three groups of Long Evans rats: EH (15min/day separation); MS (3H/day separation) on PND 2 to 14; and a control group that received standard animal husbandry (AFR). It was observed that MS animals displayed more anxiety-like behaviors (as defined by more time spent in the closed arms of the zero maze) than EH and AFR. There was no difference between the EH and AFR animals. Generally, MS animals separated 3H/day for at least 10 days tend to be more anxious than NH and AFR groups. As reviewed previously, shorter durations of parent-offspring separation associated with EH attenuates anxiety-like behaviors in response to novelty.

Some have argued that the differences across research studies on the effects of these dam-offspring separation manipulations are a result of the variability in the

methodology used (e.g., Lehmann, Stöhr & Feldon, 2000). Lehmann and Feldon (2000) in a review suggest that the duration of separation, frequency of separation, and age of pups at separation are all critical parameters when assessing the effects of maternal separation. More recent studies have also highlighted the time of the diurnal cycle in which testing during adulthood occurs as a possible confounding variable (e.g., Parfitt et al., 2007). Furthermore, Lehmann and Feldon (2000) suggest that “there appears to be a general tendency to try to infer the general effects of MS, irrespective of its form, on neuroendocrinology and behavior”. Moreover, in my review of the literature the effects of MS vary depending on the species or genera of *Muridae*. Thus, in my outline of the behavioral and physiological effects of maternal separation below, I have specified the strain and/or species where applicable.

- ***The Endocrine Effects of “Maternal Separation”***

The effects of MS on the HPA axis appear to be a direct function of the length of maternal deprivation (Levine et al., 1992; Rosenfeld, Wetmore & Levine, 1992). A minimum of 2 hours of MS appears to be necessary for a significant elevation of CORT to be observed immediately after separation (Avishai-Eliner et al., 1995 - Sprague-Dawley rats; Kuhn, Pauk & Schanberg, 1990 – unspecified species of rats; Levine et al., 1992 - Sprague-Dawley female crossed with Long-Evans male rats, Pihoker et al., 1993 - Sprague-Dawley rats) and at least 8 hours of MS is necessary for HPA axis hyper-responsiveness to occur (Cirulli et al., 1994 - CD-1 mice; Levine et al., 1992 - Sprague-Dawley female crossed with Long Evans male rats; Stanton, Guitierrez & Levine, 1988 -

Sprague-Dawley female crossed with Long-Evans male rats). Rodent pups who receive a single maternal separation (SMS) tend to have increased levels of circulating CORT immediately after separation and display an elevated ACTH response to mild stressors, such as novelty, immediately after the onset of the stressor (Levine et al., 1992 - 2, 4, 8, & 24 hour SMS; Stanton, Guitierrez & Levine, 1988 - 24 hour SMS; Stanton & Levine, 1988, 1990 - 24 hour SMS; Suchecki et al., 1993, 1995 - 24 hour SMS; all studies used Sprague-Dawley female crossed with Long-Evans male rats). It has also been reported that SMS leads to an increase of “basal” CORT and ACTH (Levine et al., 1992 - 2, 4, 8, & 24 hour SMS, Sprague-Dawley female crossed with Long-Evans male rats; Pihoker et al., 1995 - 12 & 24 hour SMS, Sprague-Dawley rats; Smith et al., 1997 - 24 hour SMS, Sprague-Dawley female crossed with Long-Evans male rats; Vasquez et al., 1996 - 24 hour SMS, Wistar rats; Vasquez, 1998 - Review Paper), and this leads to increased adrenal sensitivity (Vasquez et al., 1996, 24 hour SMS, Wistar rats).

The effects of SMS have been reported as early as PND 3 and appear to be more pronounced later in the Stress Hyporesponsive Period (SHRP) (PND 2-14) (Pihoker et al., 1993, 12 & 24 hour SMS, Sprague-Dawley rats; Suchecki et al., 1993, 24 hour SMS, Sprague-Dawley rats; Vasquez et al., 1996, 24 hour SMS, Wistar rats, Walker et al., 1991, 12 hour SMS, Sprague-Dawley rats). However, while increased behavioral responsiveness to a stressor seems to be consistent across different studies (and across different SMS manipulations), elevations of basal ACTH and CORT levels have not always been observed (Van Oers, de Kloet, & Levine, 1997, 24 hours SMS; Van Oers et

al., 1998, 24 hour SMS - both studies used Sprague-Dawley female crossed with Long Evans male rats).

As SMS subjects show an enhanced responsiveness to stress (Suchecki et al., 1995, 24 hour SMS, Sprague-Dawley female crossed with Long Evans male rats), it is generally concluded that they display poor negative feedback (Van Oers et al., 1998, 24 hour SMS, Sprague-Dawley female crossed with Long Evans male rats) due to decreased numbers of GR in the hippocampus (Vasquez, 1998, review paper). During “normal” rodent development, mineralocorticoid receptor (MR) levels in the hippocampus increase while GR levels decrease. Subsequent to a 24 hour MS, an age dependent 25-35% decrease in MR messenger ribonucleic acid (mRNA) is observed with no corresponding decrease in GR mRNA (Vasquez, 1998, review paper; Vasquez et al., 1996, 24 hour SMS, Wistar rats). Thus, SMS may alter the MR:GR ratio in the hippocampus, which may result in the increased stress sensitivity observed SMS subjects (Vasquez, 1998).

The endocrine effects of MS and MPS are less well specified than that of SMS. One hour daily MS during the stress hyporesponsive period (SHRP) appears to have a sensitization effect (i.e., elevated plasma CORT levels) on the pups (Kalinichev et al., 2002, 3 hour daily MS, Long-Evans rats). Daily MPS during the SHRP produce no differences in circulating CORT levels as compared to AFR and EH groups and higher circulating CORT than a NH group (Pryce et al., 2001, 2003, 4 hour MPS, Wistar rats). Of note here is that the endocrine effects of MPS are similar to those of the EH group. Thus, despite the more extensive separation procedure (involving longer separation time

and isolation from others compared to EH), the MPS creates a similar more robust endocrine response to stressors.

- ***The Behavioral Effects of “Maternal Separation”***

The behavioral effects of maternal separation are even more disparate than the endocrine effects. Hofer (1973b) looked at the effects of SMS for 18 hours on PND 14 in Wistar rat pups. SMS subjects displayed prolonged levels of ambulation in an open field, increased amounts of grooming, more episodes of defecation, and a delay in the onset of active sleep as compared to non-treated controls. Barna et al. (2003) report that rats removed for 24 hours on PND 9 appeared to be more anxious (lower percentage of time spent in the open arms of the plus maze) than non-separated AFR controls. These two studies are at odds with each other, as the results by Hofer (1973b) suggest that SMS produces a less “anxious” behavioral profile while the study by Barna et al. (2003) suggests the opposite. Of course, the separations occurred five days apart during the nursing period. Also, the age of testing may account for some differences. Ellenbroek et al. (1998) demonstrated that a 24 hour SMS in Wistar rats does not appear to affect sensorimotor gating (as measured by the prepulse inhibition paradigm) in prepubertal animals. However, when the same subjects were tested as adults, a clear sensorimotor gating deficit was noted.

The literature looking at the behavioral effects of SMS is riddled with paradoxical effects such as these (Lehmann & Feldon, 2000). The general consensus is that the behavioral consequences of SMS are dependent on the age of the pup at separation, the

age of testing post-separation, and the duration of the MS manipulation. In particular, the behavioral effects of 8-12 hours of SMS seems to be most effective during PND 7-14, while less stable effects are observed in younger pups, and 3 week old pups display little or no effects (Hofer, 1970,1973a).

A different picture emerges when one looks at the behavioral effects of MS. Stanton, Crofton and Lau (1992) looked at the behavioral effects of MS (6 hours daily) during PND 4-20 in Sprague-Dawley rats. No effects of MS were observed when offspring were tested as adults on motor activity (PND 13, 17, 19, 21, 29, 60), olfactory learning (PND 18) and retention (PND 25), T-maze delayed alternation (PND 23, 24), acoustic startle response (PND 23, 62), and auditory thresholds (PND 62). This finding is at odds with the previously described endocrine effects of MS. Zimmerberg and Shartrand (1992) looked at the effects of MS (6 hours daily) during PND 2-15 and the temperature of Long-Evans rats pups post separation. They demonstrated that the effects of MS were mediated by temperature and age of testing. In particular, pups that were maintained at room temperature post separation showed decreased growth and were less active in an open field at PND 16 than pups that were kept at the natal nest temperature.

The behavioral effects of MPS are also relatively ignored in the literature. Daniels et al., (2004) looked at the effects of MPS (3 hours daily during PND 2-14) in rat pups (unspecified strain) on adult behavior in the elevated plus maze at PND 60. They found that MPS subjects tended to make fewer entries into the open arms of the elevated plus maze as compared to AFR controls. Interestingly, MPS rats showed a blunted stress response (low ACTH levels, 15 minutes post stressor) as compared to AFR controls.

Romeo et al. (2003), in a similar experiment, found the same result when C57BL/6 mice were tested on an open field but only for male subjects. Wigger and Neuman (1999) have also reported a gender specific effect when assessing the effects of MPS (3 hours daily during PND 3-10) in Wistar rat pups on adult behavior. While both males and females showed increased anxiety like behavior as adults (reduction in number of entries into the open arms of the elevated plus maze) as compared to AFR controls, males also displayed an increased secretion of ACTH as a consequence of having been tested on the elevated plus maze.

Millstein and Holmes (2007) found no differences in behavior as a consequence of 3 hour daily MPS during PND 1-14 in five inbred strains of mice (129S1/SvImJ, BALB/cByJ C57BL/6J, DBA/2J, FVB/NJ) when pups were tested on PND 60 in an open-field, elevated plus maze, light/dark exploration test, and forced swim test. Parfitt et al. (2007) reported that C57BL/6 mice that experience a 3 hour daily MPS during PND 1-10 show no differences in behavior on an elevated plus maze when tested as adults. It remains unclear why these effects are so disparate across studies.

- ***Summary of the Effects of “Maternal Separation”***

A comparison of the studies mentioned reveals that none of the three categories of maternal separation appear to produce either consistent behavioral or physiological changes in either rats or mice. This inconsistency may be partly due to methodological differences in the use of the paradigm that is loosely called “maternal separation”. A 24 hour SMS early in the SHRP seems to produce the most robust endocrine effects – but

this again remains to be systematically evaluated. Interestingly, while EH appears to produce somewhat robust and consistent behavioral and physiological effects, MS does not. In particular, it appears that most MS manipulations do not affect adult “emotional” behavior. This disparity between physiology and behavior remains to be clarified. It is not sufficient to ascribe the difference to a lack of sensitivity of the behavioral test under consideration. Rather, similar to the investigation of the EH phenomenon, a systematic investigation into the mediating effects of maternal separation ought to be undertaken.

Recent Studies of Early Handling and Maternal Separation in Mice

The preponderance of this paper thus far has described relatively older studies investigating the effects of and/or the mechanisms behind early handling or maternal separation. It is therefore worthwhile to detail more recent work that employs these paradigms. There are two striking features of these more recent studies. First, there is a relatively complete shift in the model organism used in these investigations from rats to mice. As outlined previously, the prevailing school of thought has been that given “that environmental conditions have been held relatively constant or have varied in only a random fashion, the results of these studies” (i.e., strain differences within mice and differences between mice and rats on the effects of early handling and maternal separation) “have been viewed as demonstrations of a direct genetic influence upon behavior” (Ressler, 1962). Second, the reference group for assessment into the effects of early handling and maternal separation became an animal facility reared (AFR) one rather than a non-handled one (with one notable exception, Parfitt et al., 2004). In as much as

the work detailing the effects of early handling was performed using a non-handled reference group, and that the literature suggests that no differences in behavioral, physiological and neuroanatomical correlates exist between early handled and AFR subjects, this “decision” requires further empirical adjudication.

Parfitt et al. (2004) was one of the first investigators to describe the effects of early handling *and* maternal separation in C57BL/6NCrlBR mice. Male and female mice purchased from Charles River Laboratories were bred at Middlebury College to produce 24 litters. Note that the dams in this research were not reared in the lab but purchased from CRL and transported to the lab. The assumption is that such extensive transitions are irrelevant because all subjects will recover equivalently after the disturbance. Nevertheless, some labs have preferred to use as subjects, only the third generation reared in the lab. Subjects were housed in polycarbonate cages with food and water *ad libitum* on a 12-12 reverse light-dark cycle with lights off at 12:30 p.m. Temperature was maintained at 21°C. On the PND 1, litters were assigned to one of the following four treatment groups: 1) Dam and offspring were removed from the home-cage for 10 minutes daily during PND 1-10 (early handling, EH); 2) Dam and offspring were removed from the home-cage for 3 hours daily during PND 1-10 (maternal separation, MS); 3) Dam was removed from the home-cage for 3 hours daily during PND 1-10 (termed a non-handled maternal separation, NHMS) and 4) a non-handled control group (NH). Litters were maintained intact in the EH and MS groups and were placed under heat lamps for maintenance of body temperature. Subjects were weaned on PND 21,

housed with same sex littermates and thereafter received standard weekly animal husbandry.

Behavioral testing on a defensive-withdrawal task was performed only on male subjects “in order to control for the confounding effects of the females’ estrous cycle” on PND 25-29 (Parfitt et al. 2004). All testing occurred during the first four hours of the dark phase. In this test, subjects were placed inside a polyvinyl chloride (PVC) tube (20cm x 6 cm) in one corner of a brightly lit open field. Latency to exit the PVC tube and time spent outside of the tube were quantified. Both the PVC tube and the open-field were cleaned with 1% acetic acid between trials. A significant difference between groups in regards to latency to exit the PVC tube was observed. EH subjects had a significantly shorter latency to exit the PVC tube compared to MS and NH subjects. NHMS subjects displayed an intermediate response (i.e., shorter latency than NH and MS but longer than EH) and were not significantly different in terms of their latency from NH, EH or MS groups. Thus, this experiment suggests that NH and MS subjects display a similar behavioral profile in comparison to EH subjects and more importantly, that these behavioral differences are manifest relatively early in the “juvenile” period.

In a second experiment, Parfitt et al. (2004) extended the quantification of the effects described in experiment 1 to adult subjects (PND 60). Eighteen litters were bred in a manner similar to that described in experiment 1. On PND 1 litters were assigned to either an EH, MS or NH group (6 litters/group). Manipulations of the litters in each group were identical to experiment 1. Subjects were weaned on PND 21, and behavioral testing on the defensive withdrawal task occurred on PND 60 (again, only in male subjects).

Subsequent to testing on defensive withdrawal, subjects were switched from a 12/12 reversed light/dark cycle to a 12/12 light/dark cycle for testing on an acoustic stressor paradigm. This permitted for collection of corticosterone samples during the diurnal trough of the corticosterone secretion cycle. After 2 weeks of adjustment to this cycle, subjects were exposed to a 15 minute 100dB white noise stimulus. Blood samples were collected from subjects via rapid decapitation at 0, 30 or 60 minutes post exposure to stressor. It was unspecified how subjects were distributed across these post-stressor exposure groups. Blood samples were centrifuged, plasma was collected and corticosterone concentrations were determined in duplicate via a single radioimmunoassay.

No significant differences between EH, MS and NH groups were observed in the defensive withdrawal task in either of the dependent measures: latency to emerge from the PVC tube or time spent outside of the PVC tube. Interestingly, an interaction between time and group was observed in the acoustic stressor paradigm. No differences were observed between the three groups at baseline (0 minutes). However, at 30 minutes post exposure to the acoustic stressor, NH and MS subjects showed a significantly higher elevation in corticosterone levels as compared to EH subjects. Moreover, at 60 minutes post exposure to the acoustic stressor, plasma levels of corticosterone in MS subjects remained elevated and were significantly different from NH and EH subjects. Notably, at this 60 minute time point, plasma levels of corticosterone levels were not significantly different between EH and NH subjects. These data suggest that behavioral responses of subjects as a consequence of these early treatments are not persistent to adulthood;

whereas, the physiological analogues exhibit a distinct alteration of the stress response. While this discrepancy is not easily resolved, it is worth noting that EH subjects tested as adults (PND 60) in the defensive withdrawal task emerged on average 90 seconds earlier from the PVC tube and spent on average 80 seconds more time outside of the PVC tube than NH and MS subjects. The failure to find a statistical difference may have been a consequence of the unequal sample sizes across groups (NH = 25, EH = 26 & MS = 16).

Perhaps one of the most methodologically sophisticated investigations of the effects of early handling in comparison to an AFR group was performed by Millstein and Holmes (2007). In this study, five inbred strains of mice (129S1/SvImJ, BALB/cByJ, C57BL/6J, DBA/2J, FVB/NJ) were handled during PND 1-14 (i.e., during the stress hyporesponsive period (SHRP)) for 15 minutes daily, and tested on PND 60 in an open-field, elevated plus maze, light/dark exploration test, and forced swim test. Subjects were counterbalanced for sex and strain. Frequencies of maternal pup-directed behavior (arch backed nursing, licking and grooming) were also recorded for each litter post reunion. The authors note that the most stressful behavioral assay, the forced swim test, was performed at the end of the battery of tests and with at least one week between tests. The results of this experiment, as summed up by the authors, reveal “no clear and consistent effects of handling on behavioral phenotypes in any of the strains tested” (Millstein & Holmes, 2007). The only significant difference in maternal behavior was an increase in nest attendance in BALB/cByJ mice, 3 to 4 hours post reunion.

Although it is unclear why no behavioral differences in response to handling were observed, several aspects of this study may provide some insight. The first is that pups

were maintained at 32°C post separation. Thus, pups were not under any thermoregulatory distress. Hence, post reunion with the dam, they likely did not produce USVs and therefore did not elicit increased maternal responsiveness. Secondly, the AFR comparison group experienced cage changes once a week. Cage changes involve brief handling when the pups and dam are transferred to the new cage. Thus, the experiential effects of standard animal husbandry may be similar to that of handling, particularly when homeothermy is maintained during the experimental manipulations. Lastly, with the exception of BALB/cByJ mice, no differences in maternal care were observed. The difference in time spent in the natal nest was observed 3-4 hours after reunion with the dam and it is unclear what factors may have contributed to this difference. Given that 15 minutes of separation is not atypical of a disruption in bouts of maternal care (Pryce & Feldon, 2003), and that homeothermy is maintained in the pup, it is unclear in this experiment whether the pup produced any of the reunion behaviors that typically elicit increased maternal responsiveness or whether the dam responded to any change in the “stimulus properties” of the pup.

Indeed, more recent research in mice suggests that this may be the case. Parfitt et al. (2007) reported on the effects of early handling in C57BL/6 inbred mice and described them as “not as robust as initially thought”. In this study, Parfitt et al. (2007) handled C57BL/6 pups in either the first three hours or the last three hours of the light phase in the diurnal cycle. Handling occurred during PND 1-10 and pups were maintained at 28°C post separation. Subjects were tested as adults between PND 60-120 on the acoustic stressor paradigm previously described at the beginning of this section (Parfitt et al.,

2004). The authors found that animals handled in the last three hours of the light phase had lower blood plasma levels of corticosterone than those handled in the last three hours and AFR subjects. All subjects showed a characteristic HPA upregulation in response to the acoustic stressor, with plasma CORT concentrations peaking 15 min after the onset of the stressor (immediately at termination) and returning to baseline levels 90 min after the onset of the stressor. Furthermore, the characteristic blunted behavioral profile in response to novelty (as measured by number of entries into the open arms of a plus maze) was only observed in subjects handled in the last three hours of the light phase. This study highlights that the time in the diurnal cycle during which handling of the pup occurs may affect the phenotype of the pup as an adult. Furthermore, it specifies that at least in C57BL/6, the effects of early handling are not the same as those that were originally noted in rats. While it is unclear why these differences emerged, future studies looking at important mediating variables, such as changes in maternal care/responsiveness post reunion, may aid in our understanding of the manifestation of these differential phenotypes.

In summary, more recent studies suggest that as in rats, the effects of early handling appear to be as robust in mice when the comparison is a non-handled group. When the comparison is an animal facility reared group, the effects of early handling are either not manifest or variable at best. If this comparison group is to be maintained, future studies need to specify (as was done in the research using non-handled groups) the causal pathway via which differential phenotypes are manifest.

- ***Concluding Remarks***

The review of the literatures identifies several key issues that must be addressed in future research:

1) Although EH seems to result in adult offspring who are more robust in regulating challenges to their HPA system, the results for mice are more equivocal than those for rats. Are mice strains different from rat strains in their responses to the treatment manipulations? Increasingly, mice have been used in this research in order to prepare for investigations into the genetic character of the EH phenomenon. The C57BL strain has been used because it is the most common strains on which mutations are isolated for examination. The present study will use this strain to examine some of the factors that would produce variability in the effects of EH.

2) Although MS seems to result in adult offspring who are more vulnerable to challenges to their HPA system, the results are more equivocal within studies of both rats and mice. In part, this is a consequence of variations in the procedures for creating maternal separations but the variability in results across studies is present also in studies that have used the same separation procedure. If the results of the treatments on the development of the offspring's HPA regulation are mediated by patterns of maternal care, then the treatments may not be creating reliable differences in the dam's maternal care. The present study will examine the effect of treatment on the patterns of maternal care exhibited by individual dams. It is possible that there are large individual differences in maternal care within a treatment group and, if the effect of the treatment on the

offspring's development of the HPA system is mediated by maternal care, then variability across studies would result. Moreover, it would be difficult to demonstrate differences across treatment groups from one study to another. The present study will examine the relation between treatment group and the patterns of the dam's maternal care.

CHAPTER II

THE PRESENT STUDY

More recent studies in inbred mice employing a protracted period of dam/offspring separation (i.e., early handling) do not produce results that are commensurate with the previous work in rats (as noted in Chapter I); particularly when the control reference group is animal facility reared. Additionally, studies using extended periods of dam offspring separation (i.e., maternal separation for 3-6 hours/day, maternal peer separation / early deprivation for 3-6 hours/day, single 1-2 day maternal separations) suffer from a lack of consistency in behavioral, physiological and neuroanatomical correlates across studies.

This study will attempt to remove some of the variability in results by re-visiting the hypothesis that has garnered the most support: maternal mediation of subsequent offspring behavior. As in previous work, three typical disruptions of mother-offspring relations will be used: 1) Early handling (EH) - dam and pups are separated for 15 minutes daily on post-natal days 2-14; 2) Maternal separation (MS) - dam and pups are separated for 240 minutes on post-natal days 2-14; 3) Maternal Peer Separation (MPS) - dam and pups are separated for 240 minutes on post-natal days 2-14 and also pups are separated from one another. Each of these groups will be compared to a control group which will receive normal colony maintenance (Animal Facility Reared - AFR).

These four groups were selected because: 1) they demonstrate the most consistent effect of the offspring's development - EH and AFR seem to have similar effects and AFR seems to be a more appropriate control group for both MS and EH conditions; 2) the

durations of separation (daily separations of 15 minutes for EH and 4 hours for MH and MPS) seem to have the most consistent effects without compromising the homeostatic (temperature regulation, osmotic condition, etc.), nutritional status, and growth of the pup and; 3) mice, rather than rats, were selected in part for convenience but also because many studies are examining the relation of maternal and pup genotype on the consequences of the separations. C57BL/6 is a common mouse strain on which many genetic manipulations are executed. Therefore, knowledge about the contributions of disruptions of maternal care on offspring development in this strain will provide valuable information for subsequent genetic manipulations and the detailing of the neurobiological mechanism.

The review in Chapter I revealed that the manipulations of the dam-offspring relationship (short term separations and longer term separations) provide equivocal evidence of alterations in maternal care. Although the manipulations of dam-offspring contact produce equivocal results concerning the offspring's emotional development, for those studies that demonstrate an influence, maternal mediation of these effects remains the most likely explanation for the changes in the offspring's adult behavior. That is, the various forms of manipulation of dam-offspring contact (EH, MS, MPS) have been hypothesized to affect the pattern of maternal care on reunion and across the nursing period. However, the equivocal results of the separations suggest maternal manipulation may not be equivalent for all dams within each of the groups. Therefore, if the offspring's emotional development is a consequence of maternal care and this is affected only partly by the type of separation a mother-litter has experienced, then maternal care should be a better predictor of offspring emotional behavior than separation group. Thus, this study will examine the relation of maternal care, irrespective of group, to offspring development.

Specifically, this study will assess whether changes in the durations of patterns of maternal behavior that occur as a consequence of the administration of dam-pup separation treatments. Unlike previous work, the process of parental care will be analyzed using statistical techniques that will provide detailed information on potential differences in the patterns of maternal care provided during the offspring's' first two weeks among individual dams and how these individual differences relate to the treatment groups. This study will be able to differentiate between differences in maternal care upon each reunion after separation versus longer lasting differences in maternal care throughout nursing generated by the dam-pup separation manipulations. It will also attempt to replicate previous work by describing offspring emotional development in two behavioral paradigms that have been associated with the Hypothalamic Pituitary Adrenal (HPA) or stress axis; the open field and the elevated zero maze.

Currently, there are three possible explanations for why disturbing the mother-pup relationship via separations has consequences on adult emotional functioning: 1) the disruption directly affects the development of the offspring's' neural system including its hypothalamic-pituitary-adrenal (HPA) functioning; 2) the effect of the disruption is mediated via alterations of maternal behavior (because the mother has been affected by the manipulation); 3) the manipulation causes the pup to elicit differences in maternal behavior (because the pup has been affected by the manipulation). In the latter two, the effect of the disruption on the offspring's' development is mediated by patterns of maternal care. The proposed study will provide evidence to help adjudicate among these alternatives.

Hypotheses

1. It is hypothesized that individual differences in patterns of maternal care will occur, irrespective of treatment condition and that patterns of maternal care, rather than the treatment condition itself, will be a better predictor of offspring behavioral development.

This hypothesis is prompted by the variability across studies on the effects of the same treatment groups on offspring behavior. If maternal care is a mediator of offspring “emotionality”, and if manipulations of maternal care (via separation techniques) produce variability in maternal care and offspring behavior, then the treatments that define the groups may elicit certain differences in maternal care across the groups but also may mask other differences that will make the effect of the group differences less obvious. Thus, individual differences in maternal care must be examined in relation to offspring differences in behavior, irrespective of the dam’s treatment group.

2. It is hypothesized that the disruption of maternal care created by the separations produces differences in maternal care through-out the nursing period and not just at reunion.

This hypothesis is prompted by the evidence that the short-term separations (EH and AFR) have effects on the development of the offspring’s “emotional” behavior and HPA physiology. It is difficult to imagine, if the development is mediated by maternal

care, that a very short separation would produce a reunion effect strong enough to reset HPA physiology.

CHAPTER III

METHOD

General Husbandry Procedures

All animals were housed in 29x19x12cm polypropylene cages on a 14:10 light/dark cycle with lights on at 1400 hours. Temperature was kept at 21° C and humidity at 50%. Subjects were provided with food, water, nesting material and bedding of Harlan Aspen Sani-Chips approximately 1.3cm deep. Cages were changed once per week (Tuesday), between 14:00 and 15:00 unless otherwise noted.

Breeding Subjects

Ten female and five male C57BL/6 mice were purchased from Harlan Laboratories. Subjects were socially housed, same sex, 2 animals per cage, for two weeks to allow for acclimatization to the lab environment. Males were apriori, randomly assigned to be paired with each cage of two females. On day eighteen, male litter was sprinkled into each of the associated pairs of females' cage, in order to induce estrous in the females (Whitten, 1958). On day twenty one (three days later or third cage change) females were weighed, and then one male was introduced into each of the five female cages, thus creating five breeding cages. Females were checked once per week, during cage changes, for vaginal plugs and increases in weight. If either a weight gain of

2 grams or a vaginal plug was noted, that female was separated from the breeding cage, and monitored for pregnancy and parturition.

Using this strategy, these ten females each produced three litters. The first two litters were used for training purposes with students, for piloting the behavioral tests described below and for evaluation of whether extended periods of dam offspring separation (i.e., MS & MPS) may be a consequence of food deprivation. The third litter derived from the original set of animals was bred to produce experimental subjects. Breeding for three generations was done to in order to reduce or remove experimental artifacts which may have arisen as a consequence of differential rearing, husbandry and lab environments at Harlan Laboratories.

Experimental Subjects

Forty four litters were bred, across eleven cohorts, and assigned via a pseudo-random manner to one of four groups described below. Assignment was such that there was always a cohort of litters representing each of the four groups at any given time. The average litter size was six, with a minimum of four and a maximum of eight offspring. Four offspring (two male and two female) from each of the 44 litters were used in this study. The remaining offspring of these litters were used in another project. Litters from those females who produced fewer than two male and two female offspring were used for another study.

Maternal Separation Procedures

All separation procedures were performed by the same two experimenters. Dam-offspring separations occurred from postnatal day (PND) 2 to 14 (day of birth is PND 0). First, the dam was removed from the homecage and placed into a clean cage with bedding. Then, pups were removed individually from the homecage and placed into an adjacent clean cage with bedding. The clean cages were clearly labeled with a litter ID number (using an odorless Sharpie marker) in order to prevent replacement of litters into incorrect cages. After pup removal, the dam was placed back into the home-cage for the duration of the separation.

Maternal Separation (MS) pups were separated from the dam for 240 minutes (between 0900 and 1300). Maternal Peer Separation (MPS) pups were separated from the dam and their littermates for 240 minutes (between 0900 and 1300). Both MS and MPS pups were placed into a standard (29x19x12cm) polypropylene cage. For the MPS group, there were frosted plexiglass partitions within the cage to make 8 separate compartments, one for each pup (Millstein & Holmes, 2007). Prior to, and during, the 240 minute separations (Groups MPS and MS), the pup cages were placed under an infrared heat lamp adjusted to maintain the nests at 31°C ($\pm 1^\circ\text{C}$) in order to prevent cold stress. Pups in the EH group were separated from the dam for 15 minutes in the same manner as the MS group (between 12:45-13:00) but were not placed under heating lamps. All separations were ended at 13:00, one hour before lights on.

For reunion, the dam was removed again from the homecage and placed into a clean cage with bedding (the same cage used previously), the pups and then the dam were

replaced into the homecage. Holding cages and bedding for mothers and pups were not changed during the repeated separation period. An AFR control group was not separated from the dam, but received the same weekly cage changes as the other three groups.

Weekly cage changes occurred when the pups were eight days old (PND 07). The dam was removed and placed in a clean cage with bedding. Some soiled bedding from the home cage was sprinkled into a new cage and the nest from the homecage was relocated (same side/area) to this new cage. Pups were then individually placed in the relocated nest. The dam was then placed in the new homecage. This process took less than one minute.

Regular cage changes occurred on PND 7 and 14 between 1400 and 1500 hours. After PND 14, all litters were left undisturbed until weaning at PND 21. Upon weaning, subjects were socially housed 2-3 subjects per cage with their same sex, same group siblings. Cage changes continued to occur once per week thereafter.

Maternal Behavior

Maternal behaviors were observed before and after each separation period every other day from PND 2 to 14. Videorecording was performed with Panasonic WV-CP470 closed-circuit cameras (WV-LA4R5C3B lens, sensitivity of 0.1 lux at F1.4) onto a high definition (1080i) Sony Digital Video Recorder. As C57BL/6 mice predictably built nests in one cage corner, the video camera was placed 12 inches from the short side of the cage and a mirror was placed at the outside of the cage on the side where the nest was located.

Thus, front and back views (via the mirror) of the female and her litter were captured on videotape by the camera.

Pre-separation taping occurred during the dark phase, for 60 minutes between 0800 - 0900 hours for all groups. For the experimental groups, post-separation taping commenced just before (less than 5 minutes) reunion of the dam and pups then continued for sixty minutes after the dam-pup reunion. For the AFR control group, taping occurred concurrently with the other experimental groups. Thus, all tapings occurred for all groups at the same time of the light/dark cycle. All behaviors were scored by coders using Noldus Observer 5.1 on an ethogram of nursing postures and parental care behaviors (Table 1) adapted from Shoji and Kato (2006) and Stern and Johnson (1989).

Table 1

A Behavioral Ethogram of Maternal Behaviors and their descriptions

Behavior	Description
In Nest	All four feet of the dam are inside of the natal nest.
Out Nest	All four feet of the dam are outside of the natal nest.
Licking	The dam's snout is in contact with the pup(s) and concentrated head bobbing is observed.
Quiescent Nursing	The dam is in ventral contact with the pups and immobile.
Active, Not Licking	The dam is in snout contact with pups. You will see rapid random movement around various pups in the litter with her snout.

- *Adult Offspring Behavioral Measures*

All behavioral testing of the offspring occurred between PND 60 to 70 from 14:00 to 16:30, the initial portion of the light phase. One week prior to testing, each animal was tail marked with an odorless Sharpie marker so that each individual subject could be

identified. On the day of behavioral testing, all cages of animals to be tested were moved from the colony room to the behavioral testing room at 12:00. Only one cage of animals was tested at a given time and two animals in each cage were tested simultaneously (counterbalanced between the two behavioral tests of anxiety: the open field and elevated zero maze). Given that males and females were socially housed in same sex cages, either females or males were run in each counterbalanced trial. In the situation where there were three animals in a cage, the remaining one animal was tested one week later on a different behavioral measure. All other cages of animals to be tested were isolated in a corner of the room, undisturbed until testing occurred. A maximum of 12 animals were tested on any given day.

- *Elevated Zero Maze*

The zero maze consists of an elevated annular platform with two opposite closed and open arms (no center area), allowing for uninterrupted exploration. It represents a modification of the elevated plus maze model of anxiety for rodents. This apparatus was developed to eliminate the ambiguity that was associated with time spent in the center square of the elevated plus maze, as it had been demonstrated that mice spend between 20-30% of the test period in the center square (Lee & Rodgers 1990; Rodgers et al. 1992). Like the elevated plus maze, the elevated zero maze has been validated, and generates a clear and consistent behavioral profile in rats treated with anti-anxiolytics (Sheperd et al., 1994). As such, the zero maze arguably represents a better behavioral measure of “anxiety-like” behavior in rodents than the elevated plus maze.

The elevated zero maze used in this experiment consisted of a circular ring (5cm wide) with two open quadrants alternated with two closed quadrants with opaque walls 20cm high (Shepherd et al., 1994). The ring was 55cm in diameter and was elevated 60cm off the floor. Markings were drawn on the floor to ensure that the testing apparatus was maintained in the same position for every trial. Each subject was placed in the maze at the same boundary between an open and closed quadrant, with its head facing the closed quadrant and videotaped for 5 minutes in dim light conditions (20 lx) (Parfitt et al., 2007). The maze was wiped thoroughly with a solution of 90% isopropyl alcohol diluted in a 1:1 ratio with water both prior to testing and between trials.

Testing on the elevated zero maze and the open field was counterbalanced across subjects such that on the first day of testing, one male and one female from each litter were observed first on the zero maze, then on the open field. The following pharmacologically validated “anxiety-like” behaviors were scored from the videotaped performance on the zero maze: the percent time spent in the open arms and the number of entries into the open arms (Shepherd et al., 1994, Kash, Tecott, Hodge, & Baekkeskov, 1999). Noldus EthoVision XT did not provide accurate tracking in the closed arms; therefore, Noldus Observer 5.1 was used to quantify these behaviors using the ethogram described by Podhorna and Brown (2002) on the Mouse Phenome Database.

- ***Open Field***

The open field is a widely used test of locomotor behavior to characterize emotionality (Crawley & Paylor, 1997; Hall 1934), with decreased locomotion and

increased bolus production indicative of higher “anxiety”. The open field used in this study was 55cm x 55cm x 55cm. Markings were drawn on the floor to ensure that the testing apparatus was maintained in the same position for every trial. On the first day of testing, one male and one female from each litter were observed first on the open field, then on the zero maze. Subjects were placed in the center of the arena and video-recorded for 5 minutes. Subjects were further observed on each of the three days following the first test day using the same procedures, thus generating four days of open field activity. Routine cage maintenance was suspended during this four day testing period. Although exceedingly few modern studies use multiple testing on the open field, the original study by Whimby and Denenberg (1967) reported that differences in adapting to repeated exposure to this stressor provides better information about how the HPA Axis/emotion system operates differently between groups when compared to performance during a single exposure to the stressor.

The following pharmacologically validated “anxiety-like” behaviors were scored from video-records using Noldus EthoVision XT: total distance travelled, duration of time spent in center area of the field and the number of entries into the center of the open field (Carola et al., 2002). Time spent in the central area was indicative of low anxiety since this area was more open (with more light) and less “protective” (lacks tactile information about the presence of a wall) for a nocturnal animal.

- ***Setup of Noldus Ethovision XT***

In Noldus Ethovision XT, the unit of distance was (cm), the unit of rotation was degrees (°), and the unit of time was seconds (s). To calibrate the arena, a piece of pink butcher's paper with the exact dimensions of the Open Field floor (55cm x 55cm) was cut and four equal quadrants (13.75cm x 13.75cm) were clearly marked. The butcher's paper was then placed in the Open Field arena and projected via video camera to the software. This image was then used to establish the boundaries of the arena and the associated quadrants in the software.

The detection settings for Ethovision XT were selected so that both the percentage of samples in which the subject was not found and the percentage of samples skipped were less than 1% per trial. This criterion was deemed acceptable according to the EthoVision XT manual. Videotaped trials were then analyzed and the aforementioned behaviors scored. To ensure accuracy, a human observer verified the tracking of the software live as the videotapes were analyzed. Furthermore, each trial was carefully edited within Noldus Ethovision XT such that all body points (center, nose and tail) were accurately coded.

CHAPTER IV

RESULTS

Pilot data assessing food deprivation

One possible confounding variable in studies employing long periods of separation of the dam from the offspring (e.g., MS and MPS) is food deprivation. This possibility was therefore evaluated in a pilot study where individual pups from 5 litters of MS subjects and 5 litters MPS subjects were weighed (to the nearest thousandth of a gram) both pre- and post-separation during PND 2-14.

Table 2

Number of pup observations across days per treatment group.

Group	Day													Total
	2	3	4	5	6	7	8	9	10	11	12	13	14	
MS	33	30	28	28	28	28	28	28	28	28	28	28	28	371
MPS	33	33	33	33	33	33	33	33	33	33	33	33	33	429
Total	66	63	61	61	61	61	61	61	61	61	61	61	61	800

In one MS litter, three pups were found dead on PND 3, and in the same litter, 2 pups were found dead on PND 5 (c.f., Table 2). The cause of death was indeterminable but does not appear to be either a consequence of food deprivation (as milk bands were observed in all pups on the prior day) or physical maltreatment (as no easily observable evidence of this was apparent). The two surviving pups from the litter survived until adulthood, and were then used in pilot testing of other behavioral measures.

A 2x13x2 repeated measures ANOVA, with treatment condition (MS & MPS) as a between subjects factor, and day of observation (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14) and pre- vs. post- manipulation as within subject factors on litter weight yielded two main effects and an interaction. As expected pup weight increased during PND 2-14, $F(12,708) = 1654.313$, $p < 0.05$. This was qualified by a significant linear trend, $F(1,59) = 6909.240$, $p < 0.05$. In addition, pre-manipulation weight was found to be significantly different from post-manipulation weight $F(1,59) = 6909.240$, $p < 0.05$. Lastly, an interaction between day and pre- vs. post-manipulation was observed, $F(12,708) = 2.235$, $p < 0.05$. Tukey's honestly significant difference (HSD) post hoc analyses ($p < 0.05$) indicated that pre-manipulation weight was found to be significantly different from post-manipulation weight on PND 7, 8, 9, 11, 12 (Figure 1). For these days, the maximum difference in weight pre- vs. post-manipulation was 0.051 grams and the minimum difference was 0.039 grams. The differences in pup weight between MS and MPS groups were not found to be significantly different $F(1,59) = 0.004$, $p = 0.951$.

Based on these data, it was concluded that pups exposed to these extended periods of dam separation were not food deprived. It should also be noted that the subjects in this pilot study were not used in the formal study, as the additive effects of four hours of daily separation and experimenter handling during weighing were not easily dissociable.

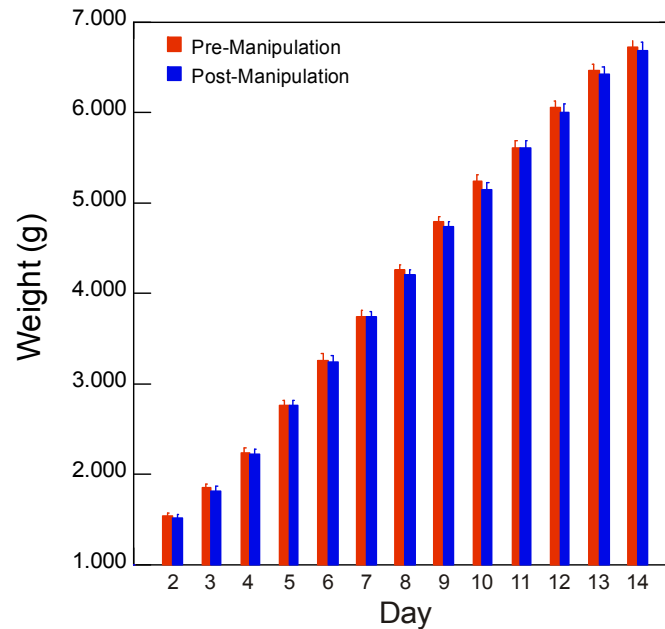


Figure 1. *Estimated marginal means of weight pre- vs. post-manipulation. Vertical lines depict standard errors of the means.*

Weaning weight

A 4 (Treatment Condition) x 2 (Sex) ANOVA assessing whether weaning weight significantly differed between treatment conditions and sex indicated a main effect of treatment condition, $F(3,311) = 21.03, p < 0.05$ and sex, $F(1,311) = 7.24, p < 0.05$ (Table 3). The interaction was not significant. Tukey's HSD post hoc analyses ($p < 0.05$) indicated that subjects in the AFR group weighed significantly less than subjects in the EH, MS and MPS groups. Additionally, subjects in the MPS group weighed significantly more than both the AFR and EH groups. As expected, male subjects weighed significantly more than female subjects.

Table 3

Estimated marginal mean weights of subjects and standard errors by Treatment Condition and Sex.

Group	Mean Weight (g)	S.E.
Animal Facility Reared (AFR)	7.28	0.12
Early Handled (EH)	7.96	0.15
Maternal Peer Separation (MPS)	8.43	0.11
Maternal Separation (MS)	8.23	0.12
Female	7.82	0.09
Male	8.13	0.09

Subject weights at Behavioral Testing between PND 60-70

Subject weights at the beginning of behavioral testing are reported separately for the zero maze and the open field, as some offspring behavioral data was lost due to equipment failure. These data are reported in Table 4 for the open field and in Table 6 for the zero maze. In addition, due to experimenter failure, offspring weight was not recorded for some subjects. These data are reported in Table 5 for the open field and in Table 7 for the zero maze.

A 4 (Treatment Group) x 2 (Sex) x 2 (Order) ANOVA assessing whether weight at the onset of behavioral testing varied as a function of treatment condition, sex or between subjects run either first or second on the open field yielded only a main effect of sex. Male subjects were found to weigh significantly more than female subjects, $F(1,113) = 57.852, p < 0.05$ (Figure 2). For reference purposes only, mean weights of subjects by treatment condition, sex and order are also presented in Figure 2.

Table 4

Number of subjects for whom behavioral data on the open field was obtained

Group	Sex	Run First	Run Second
Animal Facility Reared (AFR)	Male	7	8
	Female	7	6
Early Handled (EH)	Male	7	7
	Female	7	5
Maternal Separated (MS)	Male	9	9
	Female	10	10
Maternal Peer Separated (MPS)	Male	9	9
	Female	8	9

Table 5

Number of subjects for whom weight on the first day of testing in the open field was obtained

Group	Sex	Run First	Run Second
Animal Facility Reared (AFR)	Male	6	6
	Female	6	6
Early Handled (EH)	Male	4	3
	Female	5	5
Maternal Separated (MS)	Male	9	9
	Female	10	10
Maternal Peer Separated (MPS)	Male	9	9
	Female	8	9

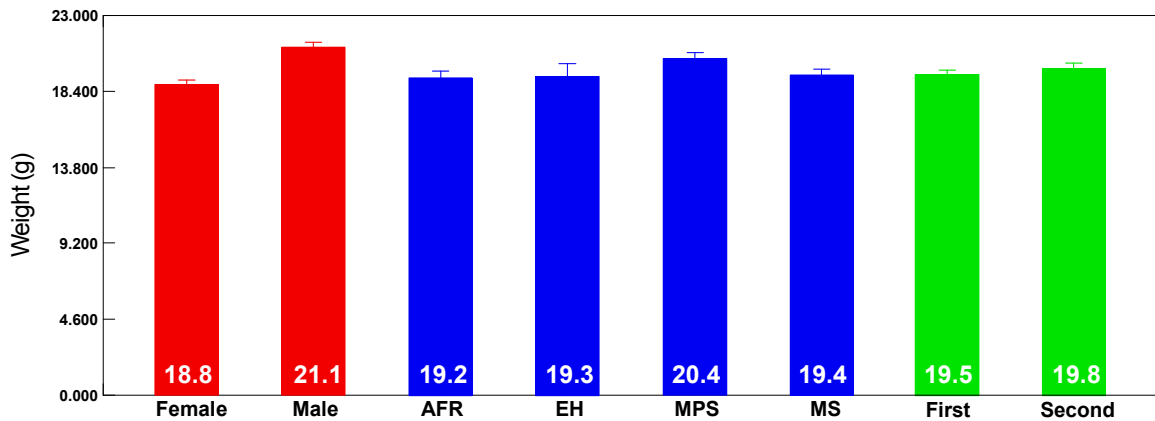


Figure 2. *Estimated marginal mean weights of subjects as a function of treatment condition, sex and order tested in the open field. Vertical lines depict standard errors of the means.*

A 4 (Treatment Group) x 2 (Sex) x 2 (Order) ANOVA assessing whether weight at the onset of testing on the zero maze varied as a function of treatment condition, sex or between subjects run either first or second on the zero maze yielded only a main effect of sex. Male subjects were found to weigh significantly more than female subjects, $F(1,82) = 44.144$, $p < 0.05$ (Figure 3). For reference purposes, mean weights of subjects by treatment condition, sex and order are also presented in Figure 3.

Table 6

Number of subjects for whom behavioral data on the zero maze was obtained

Group	Sex	Run First	Run Second
Animal Facility Reared (AFR)	Male	5	5
	Female	5	5
Early Handled (EH)	Male	5	6
	Female	5	6
Maternal Separated (MS)	Male	6	6
	Female	6	6
Maternal Peer Separated (MPS)	Male	7	7
	Female	8	8

Table 7

Number of subjects for whom weight on the first day of testing in the zero maze was obtained

Group	Sex	Run First	Run Second
Animal Facility Reared (AFR)	Male	4	4
	Female	4	4
Early Handled (EH)	Male	2	3
	Female	4	4
Maternal Separated (MS)	Male	6	6
	Female	6	6
Maternal Peer Separated (MPS)	Male	7	7
	Female	8	8

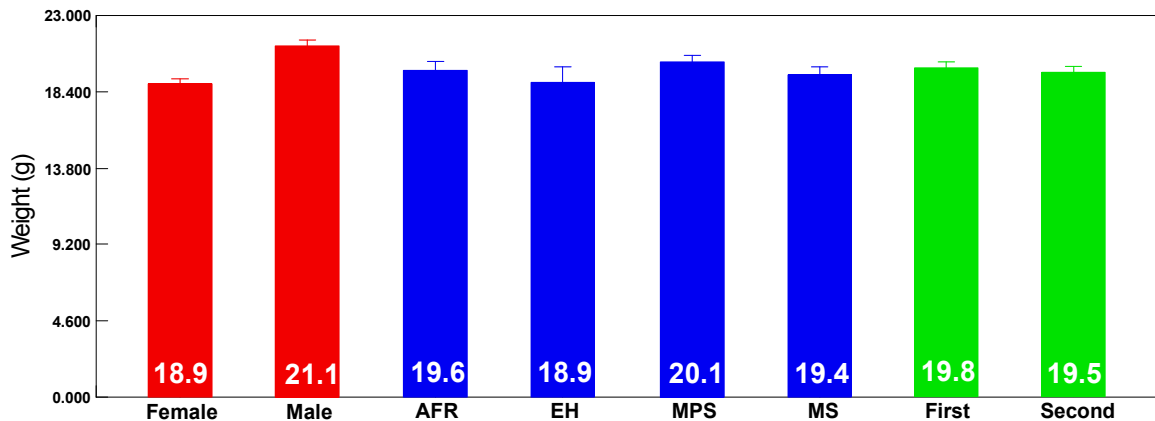


Figure 3. *Estimated marginal mean weights of subjects as a function of treatment condition, sex and order tested in the zero maze. Vertical lines depict standard errors of the means.*

Patterns of Maternal Care

Changes in maternal responsiveness as a consequence of these early treatments were evaluated next. Recall, durations of the following maternal behaviors were assessed for pups in each litter on PND 2-14: 1) In nest; 2) Quiescent nursing; 3) Active, not Licking and; 4) Licking.

These dependent measures were individually submitted to 4x7x2 ANOVAs, with separation condition (AFR, EH, MS, MPS) as a between subjects factor, and day of testing (2, 4, 6, 8, 10, 12, 14) and pre- vs. post- manipulation as a within subject factors.

- ***In Nest (Nest Attendance) Behavior***

Five significant effects were observed. A main effect of day of testing, $F(6,174) = 25.585$, $p < 0.001$ and an interaction between day of testing and treatment condition was observed, $F(18,174) = 1.801$, $p < 0.05$. Tukey's HSD post hoc analyses ($p < 0.05$)

indicated that AFR and EH groups spent significantly less time in the natal nest than MS and MPS groups over PND 2-14 (Figure 4A). Time spent in the natal nest also varied pre- vs. post- manipulation, $F(1,29) = 4.383, p < 0.05$, and by treatment condition, $F(1,29) = 11.769, p < 0.001$. A significant interaction between pre- vs. post-manipulation and treatment condition, $F(3,29) = 8.696, p < 0.001$ was also observed. Tukey's HSD post hoc analyses ($p < 0.05$) indicated that MS and MPS groups spent more time in the natal nest post-manipulation than AFR and EH groups (Figure 4B). Since the dependent measures are collected from the same individuals, a p -value of only 0.05 must be cautiously considered when concluding that the MS and MPS were different from EH and AFR groups in time spent in the nest at reunion. However, it does appear that treatment group had an effect on time in nest upon reunion, with MS and MPS dams spending more time in the nest during the reunion hour.

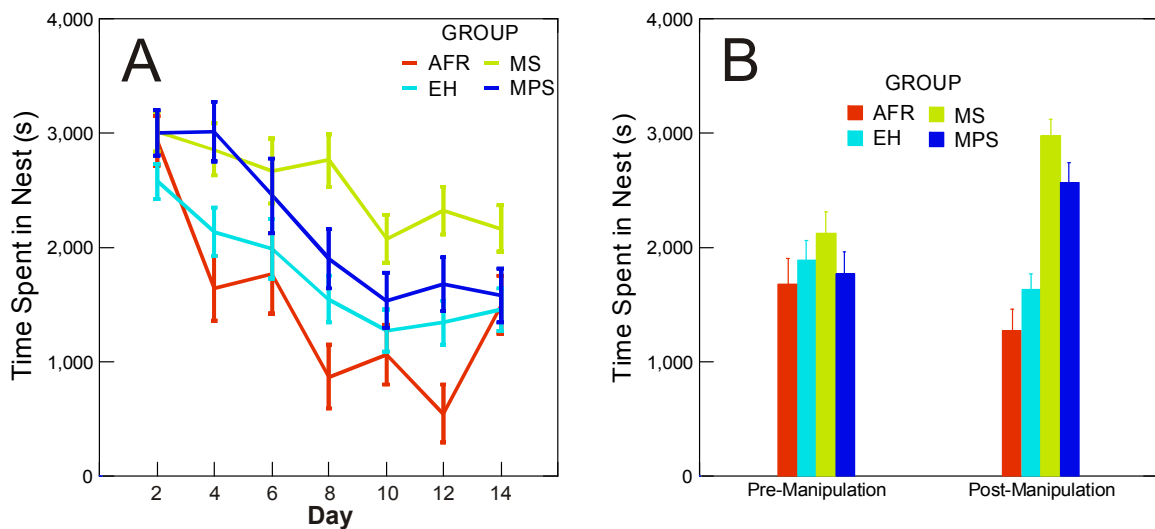


Figure 4. *Estimated marginal means of time spent in the natal nest: A) Day x Group; B) Pre- vs. Post- Manipulation x Group. Vertical lines depict standard errors of the means.*

- ***Quiescent Nursing***

Three significant effects were observed. A main effect of day of testing, $F(6,174) = 5.666, p < 0.001$ indicated that regardless of treatment condition, time spent quiescent nursing decreased between PND 2-14 (Figure 5A). This was qualified by a significant linear trend, $F(1,29) = 34.033, p < 0.001$. Time spent quiescent nursing also varied by treatment condition, $F(1,29) = 7.584, p < 0.001$. A significant interaction between pre- vs. post-manipulation and treatment condition, $F(3,29) = 3.301, p < 0.05$ was also observed. Tukey's HSD post hoc analyses ($p < 0.05$) indicated that MS and MPS groups spent more time in the natal nest post-manipulation than AFR and EH groups (Figure 5B). Again, since the dependent measures are collected from the same individuals, a p -value of only 0.05 must be considered cautiously when concluding that the MS and MPS were different from EH and AFR groups in quiescent nursing at reunion. However, it does appear that treatment group had an effect on quiescent nursing, with MS and MPS dams spending more time quiescent nursing than AFR and EH groups.

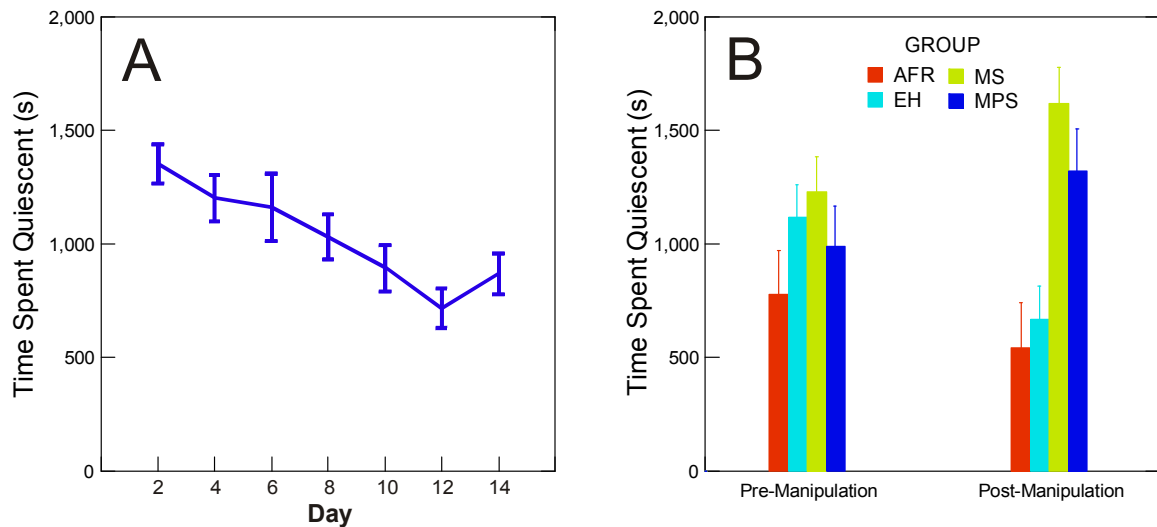


Figure 5. Estimated marginal means of time spent quiescent nursing: A) Day; B) Pre- vs. Post-Manipulation x Group. Vertical lines depict standard errors of the means.

- **Active, Not Licking**

Four significant effects were observed. A main effect of day of testing, $F(6,174) = 54.557$, $p < 0.001$ and an interaction between day of testing and treatment condition was observed, $F(18,174) = 2.964$, $p < 0.001$. Tukey's HSD post hoc analyses ($p < 0.05$) indicated that AFR and EH groups spent significantly less time active but not licking than MS and MPS groups over PND 2-14 (Figure 6A). Time spent active but not licking also varied by treatment condition, $F(1,29) = 3.505$, $p < 0.05$. A significant interaction between pre- vs. post-manipulation and treatment condition, $F(3,29) = 12.029$, $p < 0.001$ was also observed. Tukey's HSD post hoc analyses ($p < 0.05$) indicated that MS and MPS groups spent more time active, not licking post-manipulation than AFR and EH groups (Figure 6B). Since the dependent measures are collected from the same individuals, a p -value of only 0.05 must be cautiously considered when concluding that the MS and MPS were different from EH and AFR groups in time spent licking at

reunion. However, it does appear that treatment group had an effect on overall levels of activity (not licking), with MS and MPS dams spending more time active but not licking than AFR and EH groups.

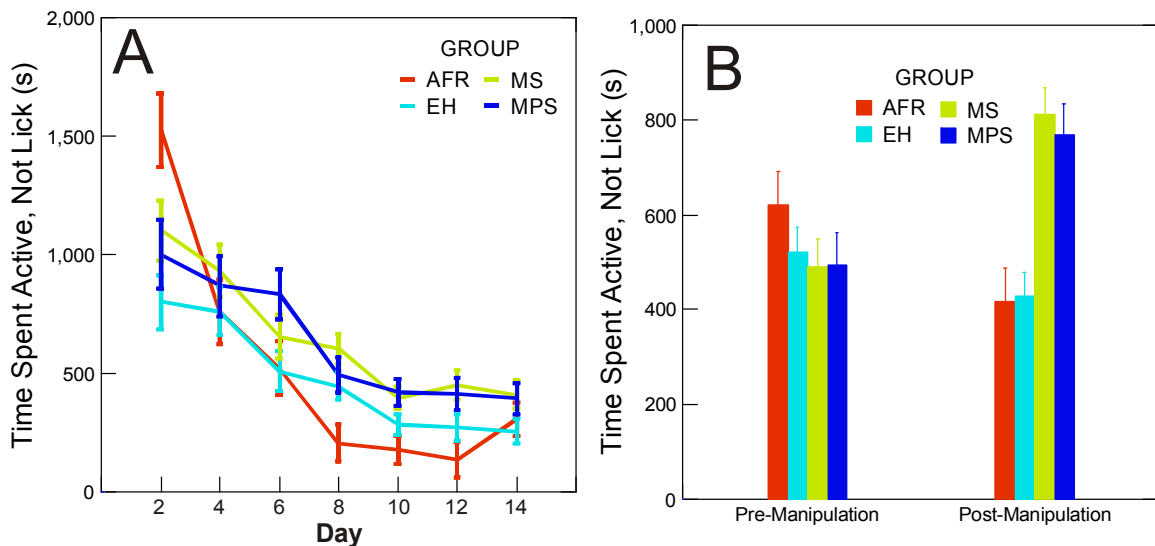


Figure 6. *Estimated marginal means of time spent active but not licking: A) Day x Group; B) Pre- vs. Post-Manipulation x Group. Vertical lines depict standard errors of the means.*

- **Licking**

Four significant effects were observed. A main effect of day of testing, $F(6,174) = 3.903$, $p < 0.05$ indicated that regardless of treatment condition, time spent licking increased between PND 2-12 (Figure 7A). Time spent licking also varied pre- vs. post-manipulation, $F(1,29) = 17.357$, $p < 0.001$, and by treatment condition, $F(3,29) = 4.440$, $p < 0.05$. A significant interaction between pre- vs. post-manipulation and treatment condition, $F(3,29) = 5.347$, $p < 0.05$ was also observed. Tukey's HSD post hoc analyses ($p < 0.05$) indicated that MS spent more time licking pre-manipulation than EH groups,

and post-manipulation MS and MPS subjects spent more time licking than AFR and EH groups (Figure 7B). Since the dependent measures are collected from the same individuals, a p -value of only 0.05 must be considered cautiously when concluding that the MS and MPS were different from EH and AFR groups in time spent licking at reunion. However, it does appear that treatment group had an effect on levels of licking, with MS and MPS dams spending more time licking post-reunion than EH and AFR groups.

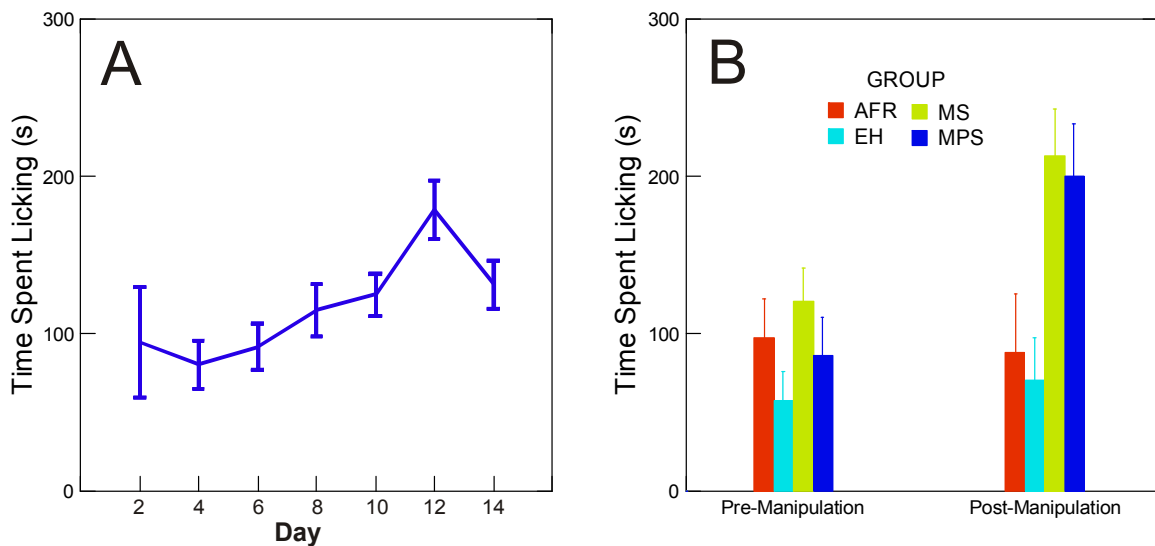


Figure 7. *Estimated marginal means of time spent licking: A) Day; B) Pre- vs. Post-Manipulation x Group. Vertical lines depict standard errors of the means.*

In general, the analyses of maternal behavior in relation to separation treatment reveal that MS and MPS treatments affected maternal behavior but these effects were restricted to the hour after reunion.

Offspring behavior in an Open Field as an Adult

The following dependent measures were collected for all subjects on each of the four days of testing in the open field: 1) Total distance travelled; 2) Time spent in the center of the open field and; 3) The frequency of entry into the center of the open field. These dependent measures were individually submitted to 2x2x4x4 ANOVAs, with sex (male, female), order run (first, second), and separation condition (AFR, EH, MS, MPS) as between subjects factors, and day of testing (1, 2, 3, 4) as a within subject factor. Again, *p*-values must be carefully considered that these dependent measures were not independent but were collected from the same individuals.

- ***Distance Travelled***

Three significant effects were observed. There was a main effect of day of testing, $F(3,327) = 116.881, p < 0.001$, indicating that the total distance travelled by subjects decreased from the first to the fourth day of testing. This was further qualified by significant linear, $F(1,109) = 217.289, p < .001$, and quadratic, $F(1,109) = 30.115, p < .001$, trends. A significant interaction between day and order, $F(3,327) = 3.585, p < 0.05$, was also observed (Figure 8A). This interaction was qualified by a significant quadratic, $F(1,109) = 6.074, p < 0.05$, and cubic trends, $F(1,109) = 8.403, p < 0.05$. Tukey's HSD post hoc analyses ($p < 0.05$) indicated that for subjects run first and second, the total distance travelled on days three and four were not significantly different, $p > .05$. A main effect of group, $F(1,109) = 4.630, p < 0.05$ was also observed. Tukey's HSD post hoc

analyses ($p < 0.05$) revealed only one significant difference: the total distance travelled by AFR subjects across days was significantly lower than MPS subjects (Figure 8B).

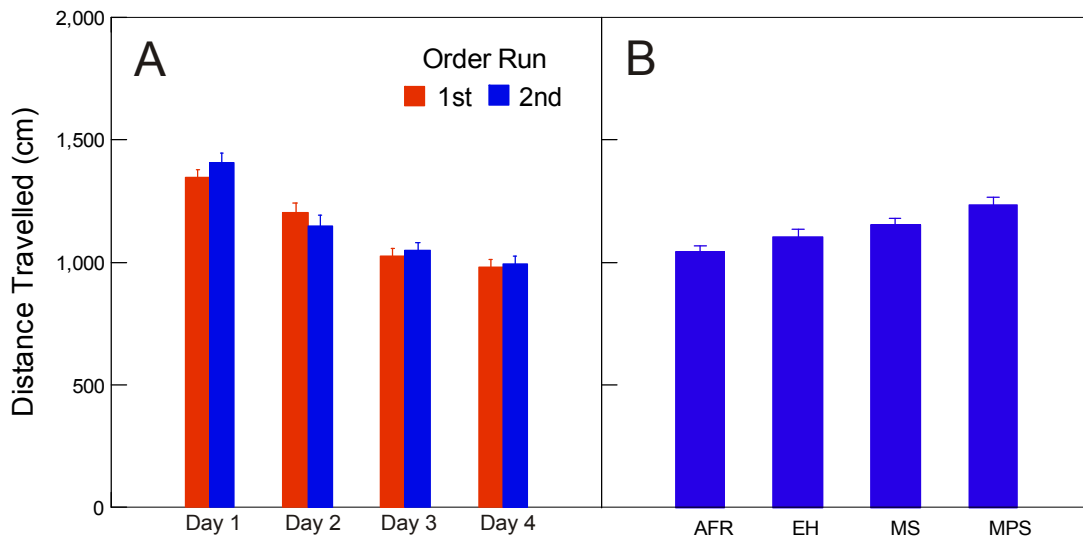


Figure 8. *Estimated marginal means of distance travelled by: A) Day x Order; B) Group. Vertical lines depict standard errors of the means.*

- ***Other Measures***

Analysis of the remaining dependent measures (time spent in the center of the open field, the frequency of entry into the center of the open field, and the frequency of entry into the area around the walls of the open field) each revealed only a main effect of day and an interaction between day and order of testing. These data are summarized in Table 8.

Table 8.

Summary of repeated measures analyses of behavior in the Open Field

Source	df	F - Statistic		
		Distance Travelled	Time Spent in Center	Frequency Entry Center
Within Subject Effects				
Day	3	116.881**	51.967**	109.680**
Day x Order	3	3.585*	4.266**	4.125**
Day x Sex	3	0.577	0.205	0.405
Day x Treatment	9	1.041	0.816	0.756
Day x Sex x Order	3	0.948	0.898	0.841
Day x Sex x Treatment	9	0.573	1.147	1.513
Day x Treatment x Order	9	0.530	1.180	1.067
Day x Sex x Treatment x Order	9	1.083	0.392	1.151
Error (Day)	327			
Between Subject Effects				
Intercept	1	3171.646**	894.917**	1326.016**
Group	3	4.63**	0.056	1.432
Order	1	0.033	0.345	0.000
Group x Order	3	0.929	1.322	1.011
Sex	1	0.149	0.140	0.002
Sex x Group	3	0.435	0.186	0.214
Sex x Order	1	0.986	3.150	0.181
Sex x Group * Order	3	0.038	0.276	0.134
Error	109			

*p < 0.05; ** p < 0.01

Recall, the open field is divided into two zones; the center and the wall. Thus, as the time spent in the center of the open field decreases, the time spent at the wall correspondingly increases. It was found that on average, the amount of time spent in the center of the open field decreased across days in a linear manner, $F(1,109) = 115.853$, $p < .001$, for the four days of testing (Figure 9).

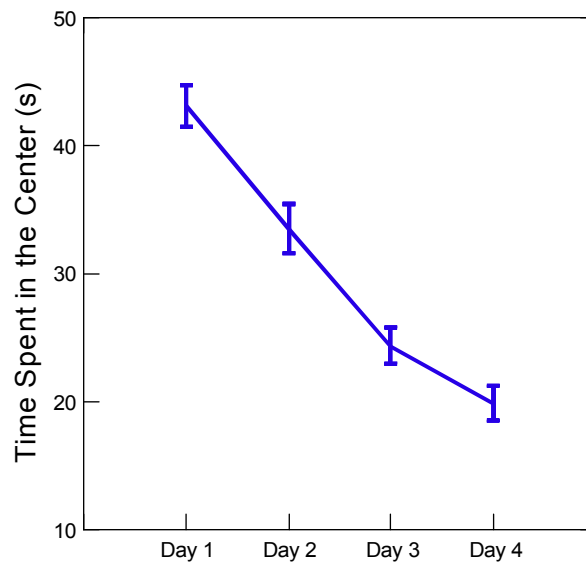


Figure 9. *Estimated marginal means illustrating change in time spent in the center of the open field across four days of testing.*

Additional Tukey's HSD post hoc analyses ($p < 0.05$) indicated that for subjects run first and second, the frequency of entries into the center of the open field were not significantly different. Further inspection of this interaction indicated that subjects that were run second on the open field on day 1 of testing, made on average 3.461 fewer entries into the wall of the open field, $p < 0.05$ and 3.445 fewer entries into the center of the open field, $p < 0.05$ (c.f., Figure 10).

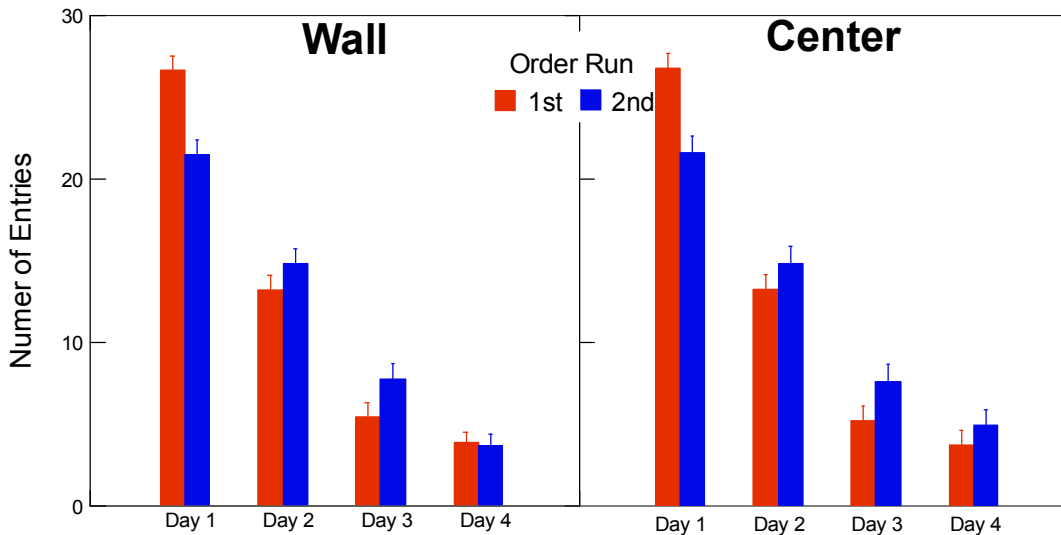


Figure 10. *Estimated marginal means illustrating differences in number of entries made into the wall or the center area of the open field across four days of testing. Vertical lines depict standard errors of the means.*

- ***Rate of change of Open Field behavior***

An alternative way of capturing the behavior in the open field would be to look at the rate of change in individual patterns of behavior across the four days of testing. To this end, each dependent measure in the open field (i.e., total distance travelled, time spent in the center of the open field, and the frequency of entry into the center of the open field) were individually regressed unto day of testing for each subject. The slope of each dependent measure was tabulated and individually submitted to a 4x2x2 ANOVA with treatment condition (AFR, EH, MS, MPS), sex (male, female) and order tested (first, second) as between subjects factors. No significant main effects or interactions were observed for any of these dependent measures (Table 9). For reference purposes only, the mean values representing the rate of change in distance travelled as a function of order run and treatment condition are presented in Figure 11.

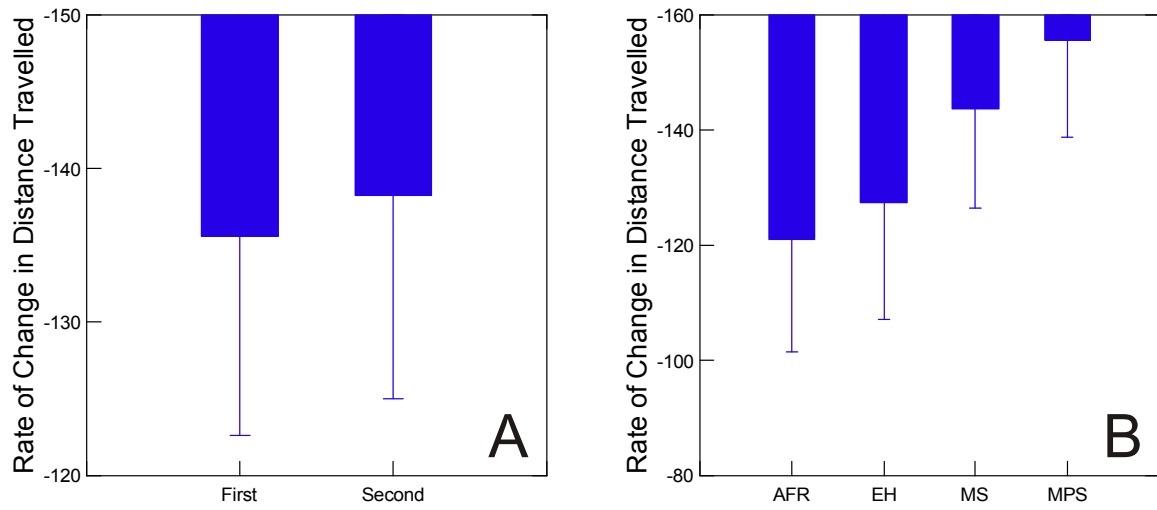


Figure 11. *Estimated marginal means illustrating rate of change in distance travelled in the open field by: A) Order Run; B) Group. Vertical lines depict standard errors of the means.*

Table 9

Summary of analyses of the rate of change of behavior in the Open Field

Source	df	F - Statistic		
		Distance Travelled	Time Spent in Center	Frequency Entry Center
Intercept	1	219.146**	102.348**	227.312**
Order	1	0.022	2.63	5.361
Sex	1	1.109	0.12	0.001
Treatment	3	0.747	1.89	0.399
Sex x Order Run	1	0.967	0.03	1.573
Treatment x Order Run	3	0.629	1.96	1.417
Treatment x Sex	3	0.258	1.36	1.937
Treatment x Sex x Order Run	3	1.035	0.07	2.102
Error	111			
Total	127			

**p < 0.01

Offspring behavior in the Zero Maze as an Adult

The following dependent measures were collected for all subjects tested in the zero maze: 1) The percent time in the open arms; 2) The number of entries into the open arms of the zero maze; 3) The number of entries into the closed arms of the zero maze..

These dependent measures were individually submitted to 2x2x4 ANOVAs, with sex (male, female); order run (first, second) and separation condition (AFR, EH, MS, MPS) as between subjects factors. No significant main effects or interactions were observed for any of these dependent measures (Table 10).

Table 10.

Summary of analyses of behavior in the Zero Maze

Source	df	% Time in Open Arms	# Open Arm Entries	# Closed Arm Entries
Corrected Model	15	0.892	1.256	1.227
Intercept	1	992.573*	1569.578*	1632.079*
Group	3	2.563	1.274	1.241
Order	1	0.232	3.596	3.747
Sex	1	0.187	0.976	0.794
Group * Order	3	0.529	0.704	0.776
Group * Sex	3	0.554	0.067	0.069
Sex * Order	1	0.274	2.237	2.101
Group * Sex * Order	3	0.671	2.011	1.852
Error	80			

*p < 0.001

Individual Differences in Maternal Care

Given that robust differences in nest attendance and maternal responsiveness that were observed between AFR and EH vs. MS and MPS conditions, it was surprising that concordant behavioral differences in the open field and the zero maze were not observed. Thus, in order to evaluate whether individual differences in maternal behavior existed between AFR and EH vs. MS and MPS groups during PND 2-14, summary scores of the four kinds of maternal behavior were plotted for each individual pre- and post-manipulation (Figure 12). Inspection of Figure 12 reveals that although MS and MPS

groups display on average higher levels of nest attendance and maternal responsiveness, substantial variability is still present in these data.

Recall that the previous evaluation of maternal behavior as a consequence of these early treatments demonstrated that changes in maternal responsiveness appear to be manifest only upon reunion with the dam. Therefore, differences in post-manipulation (POST) behavior, irrespective of day, may in part be a consequence of the concurrent upregulation of pre-manipulation (PRE) behaviors. To evaluate this hypothesis, nest attendance (NA), active but not licking (ANL), quiescent nursing (QN) and licking post-manipulation (L) were regressed unto treatment condition (GROUP: AFR & EH vs. MS & MPS), the respective pre-manipulation behavior (centered by group), and the interaction between group and centered pre-manipulation behavior. This analysis permitted for the evaluation of the *independent contributions* of each of these predictors to post-manipulation behavior. These data are summarized in Table 11.

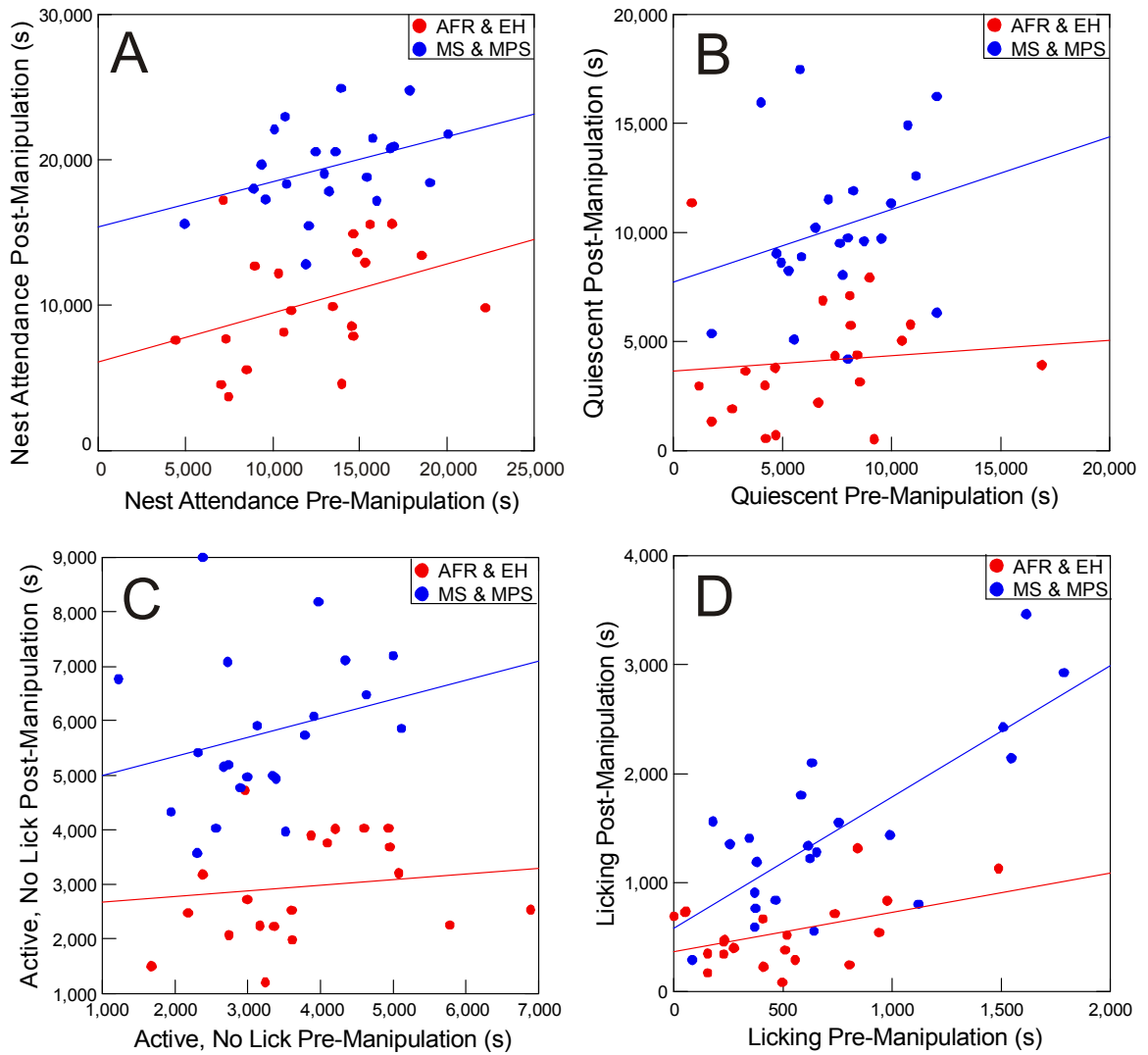


Figure 12. Differences in patterns of maternal behavior during PND 2-14, pre- and post-manipulation: A) Nest Attendance; B) Quiescent Nursing; C) Active, Not Licking; D) Licking.

Table 11

Model comparison assessing the independent contributions of treatment condition, pre-manipulation maternal behavior and the interaction between group (AFR + EH vs. MS + MPS) and pre-manipulation maternal behavior to post-manipulation maternal behavior

Model	Change in R²
NA POST = $\beta_0 + \beta_1$ GROUP	65.5*
NA POST = $\beta_0 + \beta_1$ GROUP + β_2 NA PRE	4.8*
NA POST = $\beta_0 + \beta_1$ GROUP + β_2 NA PRE + β_3 NA PRE*GROUP	0.0
Q POST = $\beta_0 + \beta_1$ GROUP	47.4*
Q POST = $\beta_0 + \beta_1$ GROUP + β_2 Q PRE	1.6
Q POST = $\beta_0 + \beta_1$ GROUP + β_2 Q PRE + β_3 Q PRE*GROUP	0.8
ANL POST = $\beta_0 + \beta_1$ GROUP	59.0*
ANL POST = $\beta_0 + \beta_1$ GROUP + β_2 ANL PRE	1.4
ANL POST = $\beta_0 + \beta_1$ GROUP + β_2 ANL PRE + β_3 ANL PRE*GROUP	0.8
L POST = $\beta_0 + \beta_1$ GROUP	37.8*
L POST = $\beta_0 + \beta_1$ GROUP + β_2 L PRE	27.4*
L POST = $\beta_0 + \beta_1$ GROUP + β_2 L PRE + β_3 L PRE*GROUP	5.5

* $p < 0.01$

As revealed in table 11, treatment condition (GROUP) accounted for a significant proportion of the variability in post-manipulation behavior in all models. For nest attendance and licking, pre-manipulation levels of behavior were found to significantly predict post-manipulation levels of behavior. A significant interaction between pre-manipulation maternal behavior and treatment condition was not observed in any of the models. Based on these data it is concluded that the up-regulation in nest attendance and maternal responsiveness that was observed post-manipulation is largely attributable to the

treatment condition. In addition, pre-manipulation levels of nest attendance and licking were found to significantly contribute to post-manipulation levels of nest attendance and licking.

As a similar pattern of results were observed for nest attendance and licking it was hypothesized that the relationship between levels of nest attendance pre- and post-manipulation may be manifest in the licking behavior of the dam. This relationship was evaluated via partial correlations which assess the relationship between two variables after controlling for the other variables of interest (c.f., Table 12).

Table 12

Partial Correlations of Nest Attendance and Licking

	NA PRE	NA POST	L PRE	L POST
NA PRE	.			
NA POST	0.20	.		
L PRE	0.44**	-0.14	.	
L POST	-0.14	0.52**	0.60**	.

Three significant relationships were observed: 1) Nest attendance pre-manipulation with licking pre-manipulation; 2) Nest attendance post-manipulation with licking post-manipulation; and 3) Licking pre-manipulation with licking post-manipulation. Since licking can only occur when the dam is in the natal nest the first two relationships are not surprising. More importantly, the third association (along with the lack of any other significant associations) indicates that the relationship between pre- and post-manipulation levels of licking is not a consequence of the relationship between pre-

and post-manipulation levels of nest attendance. Rather, the relationship seems to reflect individual differences in licking behavior.

Since an up-regulation of post-manipulation nest attendance and maternal responsiveness was observed in this study and since the literature suggests that the relationship between these early treatments to later behavior is mediated by these patterns of maternal care; offspring adult behavior was next evaluated in terms of the levels of maternal care received as pups.

The effects of Maternal Behavior on Offspring Development

As reviewed earlier in the section titled “*Contextualizing the Causal pathway in the Early Handling Phenomena*”, two key components of maternal behavior have been demonstrated to mediate the effects of early handling in rats: licking and arch backed nursing (scored as quiescent nursing in this study) (Champagne et al., 2003).

Our analysis of maternal behavior demonstrated that for the MS and MPS groups (long separation groups), there was an upregulation of nest attendance and maternal responsiveness (licking, quiescent nursing, active but not licking) post manipulation. More importantly, these differences were found to be variably manifest within treatment groups.

As nest attendance, quiescent nursing, and active but not licking were similar in pattern to each other, it was hypothesized that either nest attendance, quiescent nursing or active but not licking *post-manipulation* across PND 2-14 would be a better predictor of offspring behavioral development than the treatment condition itself. In addition, as

licking behavior was also found to be up-regulated post-manipulation and that pre-manipulation levels of licking significantly contributing to this pattern of responding; it was hypothesized that licking across PND 2-14 would be also predict offspring behavioral development.

In order to examine this hypothesis (Hypothesis 1 in the introduction), first, the correlations between nest attendance, quiescent nursing and active not licking *post manipulation*, and licking (summary of pre- and post- manipulation scores) were evaluated. These data are summarized in Table 13.

Table 13

Pairwise correlations between nest attendance and patterns of maternal responsiveness post-manipulation for each treatment group

Comparison Pair	R AFR	R EH	R MS	R MPS
Nest Attendance vs. Quiescent	0.89**	0.90**	0.95**	0.94**
Nest Attendance vs. Active, No Lick	0.49	0.58	-0.50	0.03
Nest Attendance vs. Licking	0.32	0.55	0.21	0.04
Quiescent vs. Active, No Lick	0.66*	0.46	-0.65*	-0.13
Quiescent vs. Licking	0.21	0.49	0.13	0.12
Active, No Lick vs. Licking	0.18	0.28	-0.25	-0.35

** p < 0.01, *p < 0.01

As illustrated in Table 13, regardless of treatment group, time spent in the natal nest post reunion was positively correlated with time spent quiescent nursing. Given that patterns of maternal responsiveness (quiescent nursing, active not licking, and licking) can only occur when then dam is in the natal nest (nest attendance), this result is not surprising. Furthermore this result suggests that a large proportion of the time in the natal

nest post-manipulation is spent quiescent nursing. Lastly, time spent quiescent was found to be significantly positively correlated with time spent active but not licking for the AFR (untreated) and negatively correlated for the MS groups. Thus, to a large extent, patterns of maternal responsiveness (quiescent nursing and active not licking post-manipulation, and licking) were not found to be significantly related across treatment condition. Furthermore, the maternal responsiveness data (licking, quiescent nursing, active not licking) permits for independent analysis (via single univariate tests) of patterns of maternal care on offspring behavioral development.

Next, the relationship between patterns of maternal responsiveness (quiescent nursing, active but not licking, and licking) to offspring behavior in the open field was then individually evaluated using linear regression with sex (male, female), treatment condition (AFR & EH vs. MS & MPS) and maternal responsiveness (continuous measure) and as predictors in each model. Note, each maternal responsiveness predictor was centered by treatment condition in order to assess the independent contribution of each predictor to offspring behavior. Only subjects run first on the open field were used in this analysis. Separate analyses were performed on the intercept (day 1) and slope (rate of change across four days of testing) for each of the following dependent measures: 1) total distance travelled; 2) time spent in the center of the open field and; 3) the frequency of entry into the center of the open field.

- ***Quiescent Nursing and Offspring Behavior in the Open Field***

Only one significant effect was observed (c.f., Table 14). Distance travelled on Day 1 varied as a function of quiescent nursing, $F(1,43) = 9.586$, $p < 0.01$. Subjects receiving high levels of quiescent nursing as pups, were less exploratory than subjects receiving low levels of quiescent nursing (Figure 13). Treatment condition did not predict offspring behavior in any of the models run (c.f., Table 14).

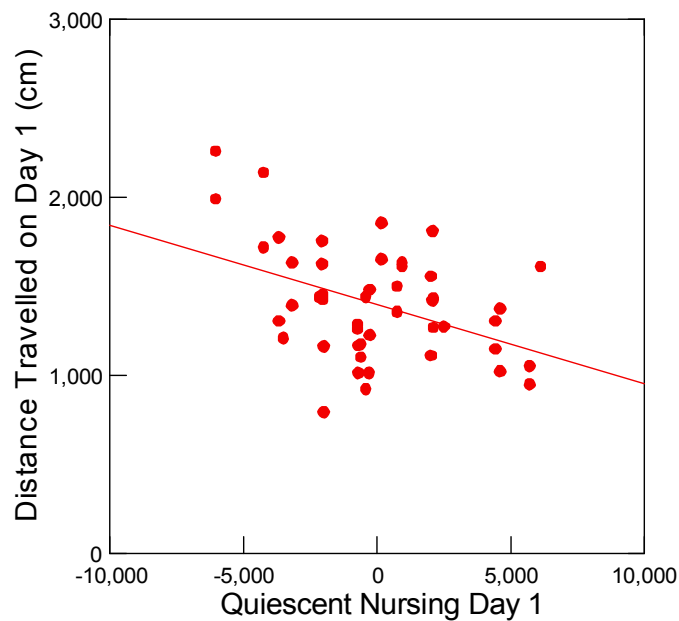


Figure 13. *Levels of Quiescent nursing predicting distance travelled in an open field on Day 1.*

- *Active, Not Licking and Offspring Behavior in the Open Field*

A similar pattern of results were observed for active not licking. Significant main effects of maternal responsiveness were observed for time spent in the center of the open field on Day 1, $F(1,43) = 5.351, p < 0.05$, and for rate of change in time spent in the center of the open field, $F(1,43) = 4.075, p < 0.05$. Subjects receiving higher levels of activity in the nest but not licking were found to spend less time in the center of the open field on Day 1 (Figure 14A) and to exhibit a slower rate of change in time spent in the center of the open field (Figure 14B). Treatment condition did not predict offspring behavior in any of the models run (c.f., Table 14).

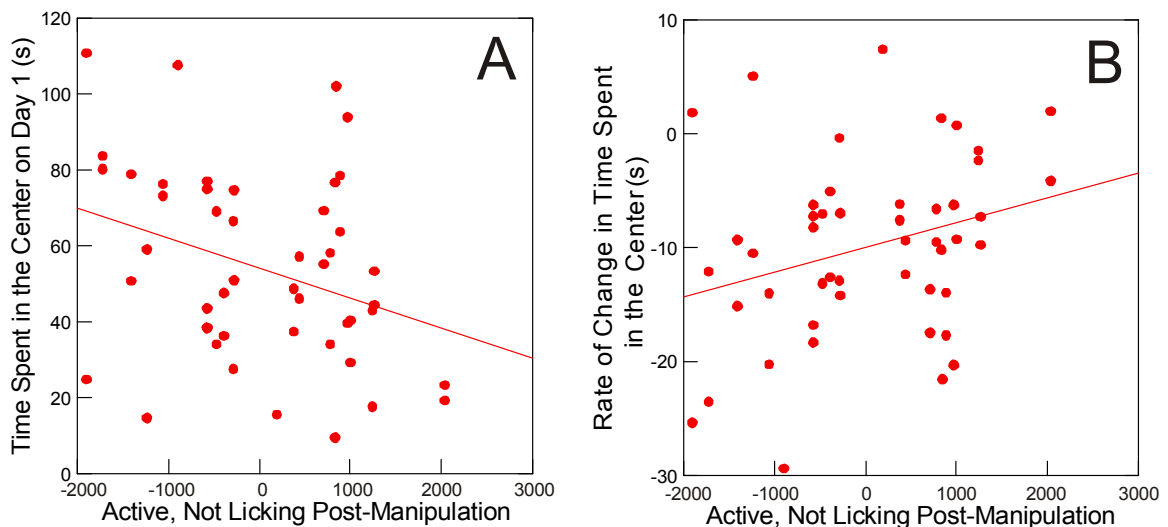


Figure 14. *Levels of Activity, but Not Licking predicting: A) Time Spent in the Center of the Open Field on Day 1; B) The rate of change in Time Spent in the Center of the Open Field.*

- ***Licking and Offspring Behavior in the Open Field***

Three significant effects were observed in regards to the relationship between licking and offspring behavior in the open field (c.f., Table 14). The frequency of entries into the center of the open field on Day 1 varied as a function of levels of licking, $F(1,43) = 4.304, p < 0.05$ and as an interaction between treatment condition and levels of licking received, $F(1,43) = 5.328, p < 0.05$. The number of entries into the center of the open field on the first day of testing was relatively constant for MS and MPS subjects regardless of levels of licking received, while subjects in the AFR & EH groups that received low levels of licking had fewer entries into the center of the open field than those that received high levels of licking (Figure 15A).

The rate of change in the number of entries into the center of the open field also varied as a function of treatment condition and levels of licking received, $F(1,43) = 6.484, p < 0.05$. The rate of change in the number of entries into the center of the open field across the four days of testing was relatively constant for MS and MPS subjects regardless of levels of licking received, while subjects in the AFR & EH groups that received low levels of licking exhibited a slower rate of change in time spent in the center of the open field than those that received high levels of licking (Figure 15B).

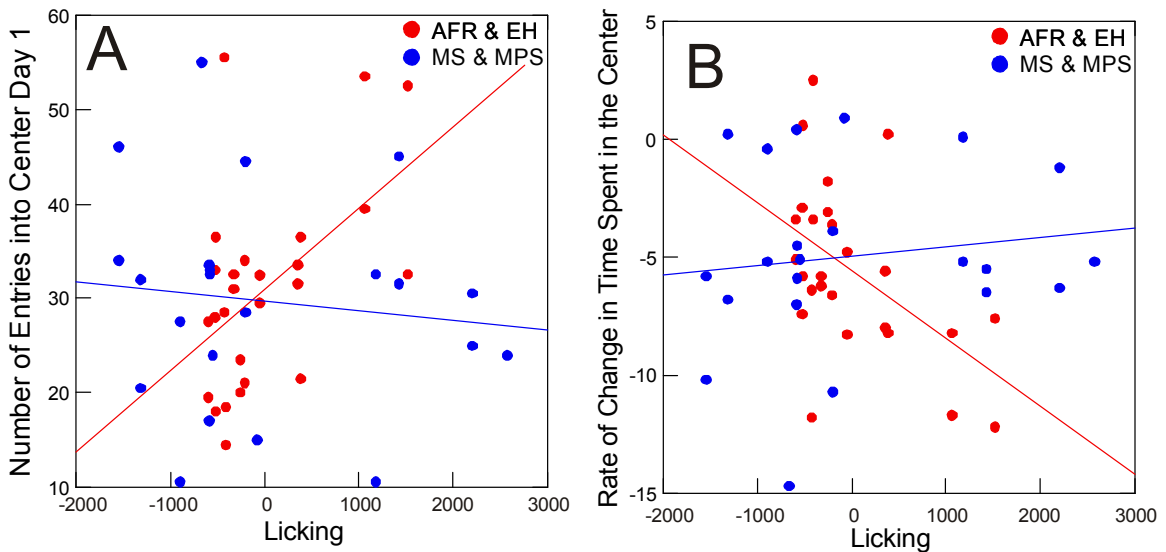


Figure 15. *Levels of licking predicting: A) Frequency of entries into the center of the open field on Day 1 by treatment condition; B) The rate of change in time spent in the Center of the Open Field by treatment condition.*

Summary of Models

The data presented on the open field in the preceding section reflect only the significant effects that were observed. Thus, a summary of the models run as well as the associated effects are presented in Table 14.

Maternal responsiveness and the Zero Maze

Recall that a large amount of data on a video cassette for the zero maze was misplaced. This, coupled with removal of subjects when creating groups according to levels of maternal behavior, reduced the sample size (between 10-15 subjects) such that power to detect significant differences between our groups was small. Thus, the relationship between maternal responsiveness (licking, quiescent nursing, active but not licking) and offspring behavior in the zero maze was not evaluated.

Table 14

Summary of analyses run for the Open Field

Model	df	Distance Travelled		Time Center		Frequency Entry Center	
		F Intercept	F Slope	F Intercept	F Slope	F Intercept	F Slope
Sex	1	0.009	0.001	0.184	0.040	0.349	0.118
Group	1	0.175	0.005	0.004	0.250	0.215	0.324
Quiescent Nursing	1	9.586**	0.443	0.338	0.149	0.122	0.260
Quiescent Nursing * Group	1	0.034	0.076	0.743	0.612	0.003	0.194
Error	43						
Sex	1	0.015	0.003	0.236	0.031	0.343	0.122
Group	1	0.240	0.003	0.001	0.317	0.169	0.337
Active, No Lick	1	0.319	0.086	5.351*	4.075*	0.068	0.198
Active, No Lick * Group	1	0.996	0.590	0.022	0.006	0.022	0.083
Error	43						
Sex	1	0.124	0.066	0.222	0.033	0.333	0.095
Group	1	0.380	0.001	0.000	0.356	0.192	0.363
Licking	1	0.411	0.678	1.562	2.430	4.304*	3.830
Licking * Group	1	3.527	2.122	0.736	1.990	6.554*	6.484*
Error	43						

* p < 0.05, ** p < 0.01

CHAPTER V

DISCUSSION

The investigation of the effects of the separation of dam and offspring on offspring development of “maladaptive” behavioral phenotypes arose in part as a consequence of a report by Bernstein (1952), in which daily handling (picked up by the experimenter for a few minutes) of infant rats led to better maze performance as adults when compared to non-handled rats. Not long thereafter, Weininger (1953, 1956) reported that handled rats were less “emotional” (as measured by “fearful” behavior in the open field) and more likely to survive severe stress as adults. Then, Levine and Otis (1958) demonstrated that the effects of early handling are maximally manifest during the first two postnatal weeks. These initial studies opened the way for investigation into the mechanism by which this early handling phenomenon is achieved.

In contrast to brief daily dam-pup separation for handling, prolonged periods of maternal separation have been reported to produce the opposite behavioral, physiological and neuro-anatomical effects associated with the early handling phenomenon (e.g., Meaney et al., 1994). However, despite what seems to be a clear and predictable relationship between early life experience and adult behavioral characteristics methodological variations have revealed paradoxical differences in observed outcomes across laboratories.

This study was designed primarily to assess the effects of early life experience, in the form of long and short periods of dam-offspring separation on the development of “anxiety-like” behaviors using the inbred strain of mouse, C57BL/6. This study assessed the following two aims:

- 1) Whether individual differences in patterns of maternal care will occur, irrespective of treatment condition and whether patterns of maternal care, rather than the treatment condition itself, will be a better predictor of offspring behavioral development. This hypothesis was formulated as the literature suggests that the effects of brief and long periods of dam-offspring separations are believed to be mediated by alterations in maternal care and that the separations produced relatively unreliable consequences on the offspring’s development.
- 2) Whether differences in maternal care stimulated by reunion of the dam and pups after separation had effects on the maternal care exhibited through-out the nursing period. This hypothesis was prompted by the inability to conceive of a mechanism by which the relatively fleeting maternal care at reunion for EH or AFR pups would be sufficient to reorganize the HPA functioning frequently reported for these manipulations.

These hypotheses were prompted by the variability across studies of the effects of the same treatments on offspring behavior. If maternal care is a mediator of offspring “emotionality”, and if manipulations of maternal care (via separation techniques) produce

variability in maternal care and offspring behavior, then the treatments that define the groups may elicit certain differences in maternal care across the groups but also may mask other differences that will make the effect of the group differences less reliable from study to study. Thus, individual differences in maternal care must be examined in relation to offspring differences in behavior, irrespective of the dam's treatment group.

It was demonstrated at weaning (PND 21), that pups that received short (EH) or long (MS & MPS) periods of separation from the dam during PND 2-14 weighed significantly more than subjects that received standard animal husbandry. Additionally, pups that were separated from the dam and then further isolated from their littermates (MPS) during PND 2-14 weighed significantly more than the group that received standard animal husbandry or those that experienced brief periods of separation from the dam (EH). These effects were not found to be persistent at adulthood (PND 60-70). Therefore, the longer separations provided by MS and MPS do not result in weight loss stress for the pups.

The patterns of maternal behavior that were observed as a consequence of the administration of these early separation treatments, revealed that the longer separation groups (MS & MPS), have an up-regulation in nest attendance and maternal responsiveness (quiescent nursing, active but not licking, and licking) during the post reunion period. However, further analyses of these patterns of behavior indicated that within treatment groups there was substantial variability in the patterns of maternal behavior exhibited to pups. Maternal care exhibited by some dams in the long separation

groups was little different from that exhibited by some subjects in the brief separation groups and vice versa.

Only one significant behavioral effect was observed when adult offspring behavior was evaluated on PND 60-70. Pups that were separated from the dam and then further isolated from their littermates (MPS) during PND 2-14 were travelled a greater distance in the open field (more exploratory) across four days of testing when compared to subjects that received standard animal husbandry (AFR). Thus, the maternal separation treatment effects had minimal influence on the pup's emotional behavior. It is important to note that significant difficulties in the use of automated tracking software were observed in this study. Future studies which use automated tracking should employ the use of infrared backlighting which renders these difficulties moot (Bailoo, Bohlen & Wahlsten, 2010).

This overall lack of an effect of treatment condition was not surprising given the more recent studies which have indicated that these separation paradigms in inbred mice (including C57BL/6) are not as robust as in rats (e.g., Millstein & Holmes, 2007; Parfitt et al., 2007), particularly when the reference or control group is animal facility reared (AFR).

It is important to address the meaning of the one significant difference (when compared to AFR, the overall activity level was much higher in the MPS group). Crawley and Paylor (1997) have indicated that in mice, overall activity in the open field will tend to decrease over time - a measure of habituation to the novelty of the open field. This study also observed this characteristic habituation to novelty regardless of treatment

condition (main effect of day of testing). Additionally, MPS subjects displayed a slower habituation response to the novelty of the open field than AFR subjects. As this difference between AFR and MPS was consistent for each of the four days of testing, no interaction between day and group was observed.

If we accept the notion that exploration and emotionality exist at opposite ends of a continuum (Hall, 1934; Whimby & Denenberg, 1967), the argument could be made that AFR subjects were found to be more emotional than MPS subjects. This result in itself is paradoxical, as the literature suggests that MPS subjects are not significantly different from AFR subjects in terms of “emotionality”. Perhaps, the animals in our study were not as stressed by the manipulations of the dam-offspring relationship as would have been predicted. Or, if slow rate of habituation to the open field represents inability to regulate anxiety, then MPS animals seem unable to regulate anxiety as well as AFR animals, with the animals from the other groups falling between the extremes of the animals from the AFR and MPS groups. What makes this interpretation paradoxical, also, is that increased activity in the open field is usually interpreted as signifying effective emotional regulation. Perhaps, MPS animals were better able to regulate their anxiety in the open field as witnessed by their activity level. Of course, another interpretation might be that activity level in the open field is not a reliable indicator of emotional regulation or of “curiosity” in novel environments. Perhaps, curiosity and anxiety are relatively independent emotions that can relate to one another in complex ways given that the open field is both a novel and stressful condition.

Given that robust differences between the combined AFR and EH versus the combined MS and MPS dams in nest attendance and maternal responsiveness, it was surprising that the predicted behavioral differences in the open field and the zero maze were not observed. Consequently, we evaluated the independent contributions of treatment condition versus patterns of maternal care, as well as the interaction between treatment condition and patterns of maternal care to offspring behavior in the open field.

Interestingly, similar effects in “emotional” behavior were observed in terms of patterns of quiescent nursing and levels of activity without licking. First, it was demonstrated that subjects receiving high levels of quiescent nursing were less exploratory (more “emotional”) on the first day of testing than those that received low levels of quiescent nursing. Furthermore subjects that received high levels of activity but not licking were more “emotional” (spent less time in center of the open field, and displayed a slower rate of change in time spent in the center) than those that received low levels of activity, but not licking. However, no effect of treatment condition was observed in any of the models run which incorporated quiescent nursing and active, not licking behavior. Thus, subjects that received high levels of quiescent nursing and activity in the nest were found to be less “emotional” than subjects that received low levels of these behaviors, irrespective of dam-pup separation condition.

A different pattern of results were observed in regards to licking behavior. A significant interaction between treatment condition and licking behavior was observed for the number of entries into the center of the open field and the rate of change in time spent in the center of the open field. The number of entries into the center of the open field on

the first day of testing as well as the rate of change in the number of entries into the center of the open field was relatively constant for MS and MPS subjects regardless of levels of licking received. Conversely subjects in the AFR & EH groups that received low levels of licking had fewer entries into the center of the open field and exhibited a slower rate of change in time spent in the center of the open field than those that received high levels of licking. If we consider the AFR/EH animals to represent some sort of control condition, then this analysis demonstrates that for AFR/EH animals, patterns of maternal licking (and not treatment group) influence performance in the open field. In contrast, animals from the longer separated treatment condition (MS & MPS) who actually experienced on average higher levels of licking, exhibit less variability in their behavioral responses to the novelty of the open field. Thus, for the longer separated groups, the patterns of licking behavior seem to be decoupled from their influence on the behavioral phenotypes that have been associated with “emotional” behavior in the open field.

The literature suggests that AFR, EH and MPS groups should display less “emotional” behavior than the MS group. In this study, it is observed that AFR and EH dams are similar to each other in their patterns of maternal care; whereas, dams in these two groups are different from MS and MPS dams (who look similar to each other in terms of their patterns of maternal care). On average, MS and MPS pups experience higher levels of quiescent nursing and activity but not licking behavior post reunion. However, this pattern of responding was found to be variably manifest within a treatment condition. Thus, it was not surprising that levels of quiescent nursing and activity but not

licking was a better predictor of offspring behavior than treatment condition itself. Furthermore, since the literature suggests that MS groups display greater emotional reactivity to stressors, and that in this study both MS and MPS pups received on average higher levels of quiescent nursing and activity, but not licking (albeit variably manifest within treatment condition), it is not surprising that offspring that received higher levels of these behaviors were found to be less “emotional” as adults.

Conversely, licking behavior represented a more pervasive form of maternal responsiveness that was strongly associated with the treatment group itself. Among maternal behaviors, only pre-manipulation levels of licking significantly predicted post-manipulation levels of licking.

Thus, as would be predicted by the notion that separation effects are mediated by their influences on maternal care, levels of maternal responsiveness are more robust in predicting offspring behavioral development than the dam-pup separation treatment condition created by the experimenter. The treatment condition has some minor effect on maternal care but it is maternal care that affects the pup’s development. Consequently, the variability in the literature concerning the effects of these treatment groups on the offspring’s development is likely due to the variability in the actual maternal care provided by the dam, which is only partially influenced by the experimental manipulation. Future studies employing these early experiential paradigms in inbred mice must examine individual differences in maternal care and its relationship to offspring behavioral development.

- *Conclusion*

At least some of the variability across studies examining the effects of early handling and maternal separation on the development of the offspring's regulation of the HPA system derive from variability in the way that the dam's maternal care is affected by the separation treatment. Thus, although the sample size of this study was insufficient to statistically assess the mediator/moderator role of maternal care on the effects of dam-pup separation on pup emotional development, the results do support the hypothesis that maternal care may be the mediator of the effects of the separation treatments on the development of the offspring. When differences in maternal care are examined, rather than treatment group, they are better predictors of the offspring's behavior. Therefore, it is proposed that patterns of maternal care are only partly influenced by the separation treatments and hence these treatments will only partially account for the offspring's development. This could be the source of the variability in effects reported across studies. Future studies should focus on individual differences among dams in their reaction to the separation treatments if the intent is to identify how the regulation of the offspring's HPA system develops.

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