Learned fear and reaction to novel stimuli: behavioral and hormonal stress responses in the maternal rat

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Abstract

Learned Fear and Reaction to Novel Stimuli: Behavioral and Hormonal Stress Responses in the Maternal Rat
Brandi Nicole Rima
MASTERS OF ARTS in Psychology
University of Richmond
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Dr. Craig Kinsley

The present thesis examines the relationship between reproductive experience and the behavioral, neural, and hormonal processes of learned fear in the female rat. Multiple research models indicate that reproductive experience functions to decrease the female’s stress response in potentially harmful environments, thus providing her with numerous survival benefits, including decreased fearfulness, increased aggression, and refined hunting skills. Based on existing understandings of maternal experience and unconditioned fear, this study was designed to determine how nulliparous (no reproductive experience, NP), primiparous (one reproductive experience, PP) and multiparous (more than one reproductive experience, MP) rats comparatively respond to a Pavlovian paradigm of learned fear, involving the pairing of a neutral stimulus (conditioned stimulus, CS) with an aversive stimulus (unconditioned stimulus, US). The behavioral and hormone analysis results confirmed several of the proposed hypotheses, thus providing further evidence that reproductive experience significantly alters the behavioral and hormonal repertoire of the female.
certify that I have read this thesis and find that, in scope and quality, it satisfies the requirements for the degree of Master of Arts.

Signature

Dr. Craig H. Kinsley, Thesis Advisor

Signature

Dr. Kelly Lambert, Thesis Advisor

Signature

Dr. Fred Kozub, Thesis Advisor
LEARNED FEAR AND REACTION TO NOVEL STIMULI: BEHAVIORAL AND HORMONAL STRESS RESPONSES IN THE MATERNAL RAT

BY

BRANDI NICOLE RIMA

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Table of Contents

I. Introduction
   Maternal Behavior
   Hormonal Mechanisms of Maternal Behavior
   Neural Mechanisms of Maternal Behavior
   Learned Fear
   Learned Fear and the Brain
   Stress Response: Anxiety
   Stress Response: Corticosterone
   Current Research Purpose and Hypotheses

II. Methods
   Subjects
   Behavioral Apparatus
   Mating Procedures
   Behavioral Procedures
   Corticosterone Sample Procedures
   Corticosterone Assay Procedures
   Tissue Preparation
   Immunohistochemical (IHC) C-fos Procedures
   Statistical Analysis

III. Results
   Behavioral Data
   Corticosterone Hormone Data
   Spearman's rho and MDS Results

IV. Discussion

V. References

VI. Table and Figure Captions

VII. Appendices
Learned Fear and Reaction to Novel Stimuli: Behavioral and Hormonal Stress Responses in the Maternal Rat

From an evolutionary perspective, reproduction is an essential process that demands large investments and often yields invaluable rewards. Darwin realized the significance of reproduction as a driving force of evolution almost a century and a half ago, and science continues to examine its effects in greater detail. Of particular interest to modern researchers is the effect of reproduction on the maternal mammal’s neural and behavioral repertoire (Kinsley, 1994; Wartella, Amory, Macbeth, McNamara, Stevens, Lambert & Kinsley, 2003; Love, Torrey, McNamara, Morgan, Banks, Hester, Glasper, DeVries, Kinsley, & Lambert, 2005).

Though reproduction affects paternal mammals to a degree, it is evident that the maternal counterpart incurs considerable changes following reproductive experience; including both behavioral and neural modifications (Kinsley, 1994). This disparity is probably due to the male and female’s differential investments in the reproductive process. Though both sexes sacrifice energy and resources in order to gain mating opportunities and achieve copulation, the mammalian female continues to invest in offspring during the subsequent events of gestation and delivery. Further, although extensive paternal behavior is observed in select species, such as hamsters and marmosets, it is a rarity amongst mammals (Numan & Insel, 2003). It is more common for the mammalian female to solely assume responsibility for the care and protection of offspring. Thus, given that the female is generally more involved in all stages of reproduction and offspring rearing, her behavioral and neural repertoire is likely more susceptible to modification following reproduction than that of her male counterpart. In fact, research suggests that the additional experiences of pregnancy, lactation, and pup
exposure are directly related to observed behavioral and neural changes in the maternal female (Wartella et al., 2003).

Plasticity is the modification of the organism’s brain and behavior in response to a changing internal or external environment (Lambert & Kinsley, 2004). Fluctuations in hormone concentrations, aging, stress, and pre- and post-natal events are examples of environmental changes that direct neural and behavioral modifications, thereby promoting plasticity (Kolb, Gibbs, & Robinson, 2003). In reference to reproduction, the female encounters new biological and environmental cues, such as pregnancy and production of offspring, all of which demand innovative survival behaviors. It is thought that the brain’s inherent plasticity allows for the development and production of these essential maternal behaviors.

Evidence suggests that the female brain prepares for such modifications early in development. Numan and Insel (2003) report shorter latencies to express maternal behavior in juvenile female rats verses adult virgin, nulliparous (NP) females. The difference in latency to exhibit maternal behaviors between juvenile and adult virgin rats implies that a developmental shift occurs at puberty. It is possible that this developmental shift prepares the female brain for future behavioral alterations that will be necessary in a new reproductive environment.

Interestingly, though, there is evidence revealing that similar behavioral and neural changes may occur in the absence of actual reproduction (conception, pregnancy, and birth). Inexperienced, NP rats exhibit maternal behaviors after an average of five to seven daily exposures to pups (Rosenblatt, 1967: as cited in Bridges & Scanlan, 2004). The observation that female rats develop a repertoire of maternal behavior without
experiencing the central events of reproduction highlights the strong influence of the
external environment on plasticity. Taken together, the findings that the female brain
undergoes a developmental shift at puberty and, in addition, maternal behavior may occur
in response to mere pup exposure suggest that the female’s brain is developed and wired
to produce maternal behavior in response to specific environmental cues.

**Maternal Behavior**

Maternal animals are inundated by environmental changes both during pregnancy
and after delivery. As a result, the maternal, plastic brain responds by producing a new
set of behaviors generally termed full maternal behavior (FMB). FMB traditionally
includes responses to pup-related cues such as gathering, grooming, crouching over, and
housing pups (Kinsley, 1994). A review of the literature indicates, though, that the
effects of reproduction extend beyond the development of FMB and include an attenuated
stress response (Wartella et al., 2003).

The additional responsibilities of protecting and caring for offspring demand that
the maternal animal is prepared and able to respond efficiently to environmental threats
and challenges. In fact, the female’s reproductive survival is dependent upon her
interaction with environmental stimuli. A critical measure of survival ability is the
organism’s response to stress, a homeostatic change in biology or behavior as a result of
exposure to a stimulus, which is sometimes aversive (Wartella et al., 2003). Empirical
examinations of female rats with differential reproductive experiences denote significant
differences in the stress response of maternal and virgin rats. More specifically,
postpartum females exhibit an attenuated stress response in comparison to NP matched
controls (Wartella et al., 2003). In an environment containing a stressful stimulus, for
example the odor of a predator, maternal rats reliably display fewer stress responses, such as freezing (a behavioral response to fear indicated by the termination of motor activity and accompanied by a characteristic posture and increased muscle tone; Fanselow, 1980), than non-maternal rats (Wartella et al., 2003; Love et al., 2005).

In addition to behavioral evidence of a decreased stress response in maternal animals, there are also findings suggesting its hormonal and neural correlates. It is thought that reproductive experience modifies the hypothalamic pituitary axis (HPA), which is implicated in the control and regulation of hormone release. Analysis of stress-provoked hormonal fluctuations in maternal and non-maternal animals provides evidence for this hypothesis. For example, circulating oxytocin concentrations increase in NP subjects after exposure to a stressful stimulus but remain unchanged in matched pregnant or lactating rats (Neumann, Torner, & Wigger, 2000). It is well understood that oxytocin is released from the HPA in response to stressors (Windle, Kershaw, Shanks, Wood, Lightman, & Ingram, 2004), thus the reported differences in oxytocin concentrations between NPs and maternal animals indicate that motherhood induces an attenuated stress response. Hence, neural and behavioral plasticity allow for the maternal animal to obtain a lessened stress response in an aversive or threatening environment.

In comparison to matched NP females, maternal rats display superior foraging and hunting abilities. When placed in an 8-arm radial maze and dry land maze individually, maternal rats learn the location of a baited food more quickly than controls (Kinsley, Madonia, Gifford, Tureski, Griffin, Lowry, Williams, Collins, McLearie, & Lambert, 1999). In addition to enhanced foraging capabilities, maternal rats also reap the benefits of superior hunting skills (Kinsley & Lambert, 2006). When a live cricket is placed in
the vicinity of the rat, the maternal rat is remarkably quicker to locate and catch the prey than the non-maternal rat (Kinsley & Lambert, unpublished data). It is likely that foraging and hunting abilities are augmented in the maternal animal so that she may find food without leaving the nest unattended for long periods of time (Kinsley et al., 1999). 

A possible explanation for significant improvements in food retrieval behavior following reproductive experience may be an increase in awareness of the surrounding environment. There is considerable evidence that the maternal female is more perceptive of her environmental surroundings, specifically social cues, than the NP rat. Fleming, Kuchera, Lee, & Winocur (1994) report that postpartum rats out perform NP controls in the completion of social learning tasks that do not involve pup or maternal cues. In a series of experiments, primiparous (PP) females were able to develop a conditioned food preference via socially mediated cues and, in addition, to complete a social recognition-learning task involving the recognition of a juvenile more adequately than control NPs (Fleming et al., 1994). It is reasoned that by being more aware of the social surroundings, maternal animals are able to respond to social stimuli with the most appropriate and useful behaviors.

Comparisons of the frequency and duration of exploratory behavior between maternal and non-maternal rats provides an explanation as to how the female becomes considerably more aware of the surrounding environment during and following reproductive events. Wartella et al. (2003) report that in an unfamiliar open field, a naturally frightening context for rats, maternal rats exhibit significantly fewer fear-related behaviors (freezes) and also more exploratory behaviors (rearing and number of blocks crossed) than non-maternal controls. Marked increases in exploratory behavior
accompany and are likely a consequence of the previously discussed attenuated stress response. Further, the maternal animal obtains pertinent social and environmental information by more actively and frequently exploring her surroundings. It is evident that such information contributes to her ability to respond to potential threats and ultimately preserve her reproductive fitness.

In an aversive or harmful environment, the maternal animal’s response to threats is critical for the protection and survival of her offspring. As explained by Kinsley and Lambert (2006), the mammalian strategy of remaining with the nest and defending offspring via aggressively responding to threats, as opposed to fleeing at the first sign of danger, was evolutionarily selected and remains reproductively beneficial. In a threatening environment involving the presence of a conspecific male or other intruder, the maternal rat exhibits heightened aggressive behaviors (Neumann, 2001; Svare, 1990; Gammie, Negron, Newman, & Rhodes, 2004). Female aggressive behavior following reproductive and maternal experience is increased and intensified to such an extent that it is commonly termed maternal aggression (Svare, 1981). This increase in aggressive behavior, like exploratory behavioral changes, is correlated to marked decreased fearfulness. Hence, maternal animals respond more aggressively possibly because their fear states are reduced.

The behavioral repertoire of the maternal rat extends beyond FMB and includes additional behaviors, which further contribute to the female’s survival and reproductive fitness. Enhanced foraging and hunting abilities, amplified awareness of the social surround, maternal aggression, decreased fearfulness, and increased exploration may be consequences of the attenuated stress response, which is provoked by reproductive and
Maternal Stress Response

Maternal experience. It should be noted that these observations often take place in the absence of pup-related stimuli and remain apparent even after pup weaning, therefore suggesting that maternal animals undergo significant and long-lasting (possibly permanent) behavioral and neural modifications (Love et al., 2005).

Hormonal Mechanisms of Maternal Behavior

Traditionally maternal behavior is divided into distinct two stages: onset of maternal behavior and maintenance of maternal behavior (Rosenblatt & Siegel, 1981). The onset of maternal behavior is regulated by the endocrine system, but may occur in the absence of hormonal regulation. The strong influence of hormones on the onset of maternal behavior was previously ascertained via blood transfusion research (Rosenblatt & Siegel, 1981). Terkel and Rosenblatt (1968, 1972) established that the induction of FMB in a non-maternal rat could be accomplished by blood transfusions from a parturient female to the virgin recipient. These findings and others denote that the hormones associated with pregnancy and lactation are, at least partially, responsible for the onset of maternal behavior. More specifically, the substantial increases during pregnancy and the ultimate declines of estrogen and progesterone concentrations in addition to other hormonal influences, such as prolactin and oxytocin, contribute to the female’s development of FMB (Bridges, 1984; Young & Insel, 2002).

As mentioned in Rosenblatt & Siegel (1981), hormone levels return to normal concentrations postpartum, thus eliminating the hormonal environment necessary for the onset of maternal behavior. While this information seems to discount a hormonal role in the maintenance of maternal behavior, it is now evident that the biological environment of pregnancy creates long-lasting changes in the female brain. These possibly permanent
alternations explain how females continue to display maternal behaviors in the absence of pregnancy-related hormonal stimuli.

**Neural Mechanisms of Maternal Behavior**

The postpartum retention of maternal behavior is accomplished via morphological and functional changes in the female brain. Though it is becoming evident that the maternal brain undergoes changes in multiple regions, early evidence of neural plasticity provoked by reproductive experience was observed in the cortex or outermost layer of the brain, a region implicated in the processing of sensory information and control of voluntary movement. By comparing the cortices of non-maternal rats maintained in an enriched environment (containing stimuli, such as exercise wheels, tunnels, and toys) and maternal rats housed in a poor environment (lacking such stimuli), it was discovered that both groups had equally complex and elaborately folded cortices (Diamond, Johnson, & Ingham, 1971). This observation revealed that reproductive experience modifies the female brain in a manner equal to that of enrichment or learning, therefore giving insight into the many benefits acquired by the maternal brain.

A second neural alteration of reproductive experience is the apparent structural modification of the medial preoptic area (mPOA) of the hypothalamus. The mPOA is designated as an essential neural site for the production and regulation of maternal behavior. Evidence of the mPOA’s involvement in maternal behavior include the termination of maternal behavior in rats following lesioning of mPOA neurons (Numan, 1974; Numan, Corodimas, Numan, Factor, & Piers, 1988; Numan, McSparren, & Numan, 1990) and, in addition, the induction of maternal behavior via implanting estrogen into the mPOA region (Numan, Rosenblatt, & Komisaruk, 1977; Numan et al.,
Interestingly, in addition to its integral role in the production of maternal behavior, research investigations have also ascertained that the mPOA undergoes plastic changes in response to reproductive experience, more specifically pregnancy. Modifications of the mPOA during pregnancy include significant increases in the volume of cell bodies, length of dendrites, and number of dendrites (Keyser, Stafisso-Sandoz, Gerecke, Jasnow, Nightingale, Lambert, Gatewood, & Kinsley, 2001). Treatments of pregnancy hormones, specifically progesterone and estradiol, to non-pregnant rats induced the same effects in the mPOA (Keyser et al., 2001; Kinsley & Lambert, 2006), thus providing further evidence that the hormonal environment of pregnancy produces the neural changes associated with maternal behavior. Modifications of the mPOA during pregnancy are also responsible for the immediate expression of maternal behavior following delivery and, thus, function to prepare the female for the additional, imminent demands of her offspring.

Another important neural consequence of reproductive experience concerns observable changes in memory function. Maternal animals are characterized by their development of maternal memory, the long-term retention of maternal behavior instituted by exposure to pups and prior maternal experience (Bridges & Scanlan, 2004). The experimental finding that lesions applied to the nucleus accumbens disrupt maternal memory designates the structure as a player in the formation of maternal memory (Lee, Li, Watchus, & Fleming, 1999; Li & Fleming, 2003). As explained by Bridges and Scanlan (2004), the nucleus accumbens communicates via neural projections with other regions of the brain, including the mPOA, olfactory and central amygdala, and the bed nucleus of the stria terminalis, which are all involved in the production and expression of
maternal behavior. It is evident that the nucleus accumbens' regulation of maternal
memory is imperative for reproductive survival, yet further research findings indicate that
other forms of memory are equally important for the maternal female.

In particular, the female’s spatial memory capabilities contribute to her ability to
care for and protect offspring. In a series of experiments, Kinsley et al. (1999) observed
greater spatial ability in parous female rats versus NP controls. It is reasoned that
enhanced spatial memory helps the maternal animal to adequately remember the
surrounding environment so that she may search for food and return to the nest in a
minimal amount of time, thus diminishing potential dangers to her pups (Kinsley et al.,
1999).

Aside from the spatial environment, it is beneficial for the maternal animal to
comprehend and remember other aspects of the environment, for example the presence of
a predator. In conjunction with the idea that pregnancy and lactation decrease the
animal’s level of fearfulness, we are also interested in the effect of reproduction on the
learning and memory of fear. It seems logical that a maternal animal benefits from first
being able to recognize a fearful stimulus and second forming a memory of the stimulus
in relation to the fear emotion.

Learned Fear

Though it is apparent that maternal rats develop a reduced state of natural fear, to
our knowledge, research has not yet determined the effects of reproduction on learned
fear. Learned fear is distinct from natural fear in that the animal does not innately fear
the stimulus. In contrast, the animal learns to fear the naturally unthreatening stimulus
or context via its association with a painful or aversive stimulus. Learned fear is
traditionally studied using two forms of the Pavlovian conditioning model: contextual fear conditioning and classical fear conditioning.

Contextual fear conditioning involves training animals to fear a previously harmless contextual setting, for example a chamber with a textured floor, via its association with a threatening stimulus, such as the odor of a predator. Typically, the animal is placed in the contextual specific chamber and exposed to the fearful stimulus over a number of trials. By the final trial, the animal presumably learns that the contextual setting includes and predicts the aversive stimulus. The expression of fear behaviors (for example, freezing, defecation, fear-potentiated startle, changes in heart rate, etc.) is likely to continue in the contextual environment even in the absence of the aversive stimulus. In the past, learned fear has been established in a variety of contextual fear conditioning paradigms (McGregor, Staples, Cornish, & Hunt, 2004 under review).

Learned fear can also be examined under a classical fear-conditioning paradigm (McAllister & McAllister, 1971). Classical conditioning involves the presentation of a neutral stimulus (conditioned stimulus or CS), for example, a tone or a light, followed by the occurrence of an aversive stimulus (unconditioned stimulus or US). The animal will express fearful behavior once the CS and US have been paired. In other words, the animal presumably learns that the presentation of the CS predicts the US (the light turning on signals or predicts a danger).

Commonly, the CS and US are presented by trace, delay, or long delay procedures. Trace conditioning includes a time gap between the presentation of the CS and that of the US. The delay conditioning requires the US to be presented directly after the termination of the CS without a time gap. The trace and delay procedures not only
differ in terms of the presence or absence of a trace interval, but also in the inter-stimulus interval (ISI), or CS-US delay. In order to compensate for this difference between trace and delay conditioning, some argue that long delay procedures should be implemented (e.g., Ivkovich, Paczkowski, & Stanton, 2000; Beylin et al., 2001). Here, the US directly follows the CS as in delay conditioning but the duration of the CS is lengthened to equal the entire ISI duration. Regardless of the type of conditioning procedure employed, classical fear conditioning gives the researcher the ability to measure the acquisition (learning) and retention (memory) of fear.

Learned Fear and the Brain

The neural circuitry of learned fear is of particular interest to researchers because learned fear processes include multiple cognitive functions including predicting, representing, and defining relationships between events (Rosen, 2004). Research literature discussing the neural correlates of learned fear is vast and, further, agrees that the amygdala is the central brain structure responsible for Pavlovian learned fear. In fact, bilateral damage to the amygdala seriously impairs Pavlovian fear conditioning (Blair et al., 2005) indicating a critical relationship between learned fear and the amygdala.

Together thirteen main nuclei and their respective subnuclei form the amygdala (Pitkanen, 2000), two of which play key roles in the processing of Pavlovian learned fear. Though their specific roles differ, both the basolateral nucleus (BLA) and central nucleus (ACE) of the amygdala are involved in Pavlovian learned fear processes. The BLA, composed of the lateral, basal, and accessory basal nuclei (Rosen, 2004), is implicated in the animal’s ability to learn to fear the neutral stimulus. Cells within the BLA exhibit altered activity following CS-US pairings (LeDoux 1993; see also Davis, 1992),
supporting the accepted conclusion that the BLA region receives sensory information about the CS and US. Once the BLA obtains such information, it commands other regions to produce fear-related behaviors (Rosen, 2004).

The lateral, basal, and accessory basal subnuclei of the BLA project to the ACE, which is composed of the capsular, lateral, and medial parts (Rosen, 2004). The ACE receives commands from the BLA to initiate the expression of fear behaviors. Early research investigations revealed that ongoing behavior is terminated when the ACE is electrically stimulated (Davis, 1992) thus uncovering the critical role of the central nucleus in the manifestation of fear-related behaviors. Freezing, the cessation of ongoing behavior, is observed in multiple animal species as a response to, and in anticipation of, a threatening stimulus (Rosen, 2004). Though a functioning ACE is imperative in order for the animal to freeze in response to a US-CS pairing, it is evident that other regions are also involved in the production of fear-related behavior. The ACE maintains projections to several nuclei of the midbrain and brainstem (Davis, 1992; Rosen, 2004). These projections function to coordinate all behavioral, autonomic, and endocrine responses in the harmful environment (Rosen, 2004). In conclusion the neural circuitry of learned fear, in terms of acquisition of the CS-US pairing and the production of fear-related behavior responses, is documented and understood to include two nuclei of the amygdala, the BLA and ACE, and their respective projections.

While it is clear that the amygdala plays a significant role in the association of the CS with the US and the production of fear-related behaviors, it is not certain how it functions to create and store the fear memory representation during conditioning. Most studies advocate that the amygdala modulates memory storage (Cahill & McGaugh,
The term memory modulator refers to the amygdala's ability to advance recall and recognition by combining an emotional feeling with the memory and communicating this information to other neural regions (Huff & Rudy, 2004). Considering the role of the memory modulator, it is not surprising that memory performance is not completely destroyed when lesions are made to the amygdala (Sutherland & McDonald, 1990). Instead, amgdala lesions result in the loss of the emotional content related to the memory. Though the amygdala is not the central structure liable for memory storage (Huff & Rudy, 2004), the structure aids in enhancing memory capabilities particularly when the events occur in an emotionally arousing context (Huff & Rudy, 2004). Besides assigning the amygdala the role of memory modulator, modern research cannot account for any other details of the amygdala's involvement in memory functioning.

The neuronal structure that is most critical in creating, storing, and retrieving memory representations is the hippocampus (Rudy, Barrientos, and O'Reilly, 2002). Though some memory representations are independent of the hippocampus, most research studies agree that the memory processes involved in trace fear conditioning are dependent upon the hippocampus (McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998). It is generally understood that pathways between the amygdala and the hippocampus are necessary in situations in which the CS and US are separated in time (McEchron et al., 1998; Quinn, Oommen, Morrison, & Fanselow, 2002). The idea is that the time gap between the presentations of the CS and the US requires a memory representation of the CS, and the hippocampus completes the task of creating and maintaining the representation (Rodriguez & Levy, 2001; Solomon, Vander Schaaf, Thompson, & Weisz, 1986). This conclusion is derived from the observation that
hippocampal lesions eliminate learning behavior under the trace conditioning treatment (Ivkovich & Stanton, 2001; McEchron et al., 1998; and Solomon et al., 1986). Further, experimental manipulations that increase hippocampal function consequently result in a substantial improvement and increases in fear behavioral responses in a trace-conditioning paradigm. For example, hippocampal stimulation via the administration of physostigmine, an acetylcholinesterase inhibitor, promotes a significant increase in freezing behavior in rats following trace conditioning (Moye & Rudy, 1987; see also Castro, Paylor, Moye, & Rudy, 1990; Moye & Vanderryn, 1988). It should be noted, however, that this pattern of results is not obtained when using the delay conditioning procedure (Kaneko & Thompson, 1997) indicating that the neural processing of the different types of fear conditioning are not identical.

**The Stress Response: Anxiety**

Fear is characterized as an emotional state in response to a specific stimulus, whereas anxiety is a general state of uneasiness that may persist in the absence of specific stimuli. Though the definition of fear differs from that of anxiety, the two emotional states are marked by similar symptoms and may occur simultaneously (Davis, 1992). It is logical to assume that an environment or situation that elicits unconditioned or conditioned fear is also provoking a sense of anxiety for the rat. In fact, drug research indicates that the amygdala, the primary neural site responsible for the processing of fear, is also implicated in that of anxiety (Davis, 1992). Thus, behavioral measures of anxiety, such as scratching and self-grooming, are helpful when determining the degree of experienced fear and anxiety.

**The Stress Response: Corticosterone**
The animal’s state of fear and anxiety can also be established via the hormonal stress response. Stressors, such as an approaching predator or being placed in an unsecured open area, trigger the rodent’s hypothalamus to release corticotrophin-releasing hormone (CRH), which, through a series of steps, stimulates the release of the glucocorticoid corticosterone from the adrenal gland (Sapolsky, 2002). Thus, high corticosterone concentrations signal that the animal is stressed. Glucocorticoid assays have been previously used and validated in other research investigations of the stress response in several species, including primates and rats (see Bardi, Bode, Ramirez, & Brent, 2005; Millspaugh & Washburn, 2004).

Current Research Purpose and Hypotheses

To our knowledge, research literature elucidating a relationship or lack thereof between maternal experience and changes in the Pavlovian learned fear process is presently limited. Examinations of unconditioned fear and maternal experience suggest that an attenuated stress response and an overall decrease in fearfulness provides numerous survival benefits, such as enhanced and increased hunting and gathering skills, exploration, social awareness, and aggression, to the female rat. In comparison to unconditioned fear, learned fear processes are equally as important for survival. The animal’s environment is always subject to change, thus the potential to encounter novel and possibly dangerous stimuli is great. Since survivability is dependent upon the prediction and appropriate response to threatening stimuli, Pavlovian conditioning research models provide pertinent information about the animal’s fitness (Rosen, 2004).

Every animal, regardless of species, gender, or reproductive history, must competently respond to a changing environment in order to survive; yet the maternal
animal carries the additional weight of protecting her reproductive fitness. It is therefore reasonable to predict that the maternal versus non-maternal animal is more behaviorally and biologically equipped to cope in and respond to a Pavlovian fear-conditioning model. Thus, the overall purpose of the current research study is to determine if maternal rats, and non-maternal controls, will express a similar relative pattern of fear both behaviorally and biologically under a Pavlovian fear-conditioning paradigm, as compared to unconditioned fear.

Based on the information provided by studies relating maternal experience to natural or unconditioned fear, we propose several hypotheses. First, it is hypothesized that maternal subjects (PP and MP groups) will express fewer fear-related (freezing) behaviors, measured by frequency and duration, than non-maternal subjects (NP group) during the retention/testing trials of the Pavlovian conditioning model. It is also predicted that PP and MP groups will explore the conditioning chamber more frequently and for longer durations during the retention/testing trials than the NP group. We further hypothesize that the maternal groups will express anxiety-related behaviors less frequently and for shorter durations (scratching and self-grooming) than the non-maternal group during the retention/testing trials. In addition, it is proposed that the NP group versus the PP and MP groups will exhibit significantly higher corticosterone concentrations following the fear conditioning training trials. Finally, we hypothesize that the PP and MP groups will display less c-fos activation in the amygdala and hippocampus following Pavlovian fear conditioning.

Under the assumption that the female’s brain and behavior are continually modified with each new reproductive experience, it is expected that there will also be
significant differences between the two maternal groups across all dependent measures. It is hypothesized that the MP group will display fewer fear-related (freezing) and anxiety-related (scratching and self-grooming) behaviors, measured by frequency and duration, than the PP subjects. We also propose that the MP group will explore more frequently and for longer durations than the PP group. It is anticipated that, following the fear conditioning training, the MP animals will maintain lower corticosterone concentrations than the PP group. Finally, we hypothesize that less c-fos expression will be measured in the MP versus PP animals.

The accomplishment of the current research goals and investigation of the above hypotheses will contribute to scientific understandings of the relationship between maternal experience and learned fear.

Method

Subjects

Twenty-eight female and five male Sprague-Dawley rats from the University of Richmond Animal Facility were employed in this study. At weaning age, each female animal was assigned to either the NP (n=10), PP (n=9), or MP (n=9) group. Male animals were stud males previously mated from the facility.

Regardless of group, each female animal was kept in a home cage with human disruptions limited to feeding and cleaning of cages. PP and MP groups were housed singly during pregnancy and with pups during the period after birth and before weaning at 21 ± 3 days after delivery. After pup weaning, MP and PP animals were housed singly until testing. NP animals were doubly housed until approximately one week before testing at which time they were singly housed. All rats were housed in 20 x 45 x 25 cm
clear polypropylene cages. The bottoms of the cages were covered with corn cob bedding, and the tops were wire lids. Food (Harlan Tekland, Indianapolis, IN, Global 18% Protein Rodent Diet, product # 2018) and water were available ad libitum. The animal housing room was kept on a 14:10 light/dark cycle with lights on at 0600h. The Institutional Animal Care and Use Committee (IACUC) of the University of Richmond have approved procedures pertaining to all animals in this study.

**Behavioral Apparatus**

The classical conditioning training chamber consisted of a glass aquarium (50.8 X 25.4 X 30.48 cm) with a wire lid. The light source, a white light bulb (17 W 120 v), a red light (25 W 130 v), and a CD player (Sony CD Dream Machine Clock Radio) with attached speakers (Altec Lansing Powered Audio System Product # VS2220) located outside of the chamber. The white and red lights were placed within approximately six inches of the chamber. The white light was turned on and off by an adapter (Lutron 300 watt White Attache Tapletop Lamp Dimmer). The speakers were placed on top of the aquarium lid and turned to the maximum volume. A burned CD was used to emit the aversive noise, a hawk cry (26kd) retrieved from [http://www.animalpicturesarchive.com/animal/SOUND/](http://www.animalpicturesarchive.com/animal/SOUND/), at specific times.

The glass chamber, white light, and red light were also used during the testing trials. Additionally, nine toys were located inside the chamber (5 small balls, 2 plastic tubes, 1 cylinder with a bell, and 1 round tube). A video camera was used to record the behaviors.

**Mating Procedures**

Once the animals were acquired from litters in the University of Richmond
Animal Facility, they were separated into groups and mated accordingly. All animals were housed and maintained in standard cages until the time of testing. Eight of the NP rats began testing at approximately 95 days of age. The remaining two NP animals were maintained until approximately 160 days of age before testing. At approximately 50 days of age, the PP animals were mated with stud males. After pup weaning (21±3 days after delivery), the PP animals were maintained according to standard procedures until testing (approximately 110 days of age). The MP rats underwent the same treatment, but they were mated and delivered twice. The MP animals were mated for the first time between 50 and 100 days of age. The second mating took place after weaning of the first litter. After the second pup weaning, MP animals were housed and maintained according to standard procedures until testing (approximately 200 days of age).

**Behavioral Procedures**

Training and testing procedures took place over the period of two consecutive days in an isolated observation room. In addition, all behavioral procedures were conducted during the light phase of the rodent’s light/dark cycle. Though the exact time of the experiment varied for each subject the tests began at a time between the approximate hours of 8:00 AM and 4:00 PM.

On day one of the experiment, classical fear conditioning training was completed. First, each animal was placed individually into the dark testing chamber with dim light provided for the experimenter by the red light. A 10 min adaptation period took place before the start of conditioning. Following the adaptation period, the US (aversive noise) and CS (white light) presentations began. The light (3 sec) and hawk cry (1 sec, 100 db) stimuli were presented with an inter-stimulus interval (ISI) of 10 sec and random inter-
trial intervals (ITI) ranging between 5 and 181 sec (Table 1).

A trained student presented the light stimulus specific, predetermined times (10 sec. after the termination of the noise). The timing of the US-CS presentations was scheduled and practiced prior to testing day. Conditioning included a total of 13 CS-US trials. After the conditioning session, each animal was returned to the home cage and the conditioning chamber walls and floor were cleaned with 95 % ethanol.

Approximately 24 hr after the start of the training, testing procedures were conducted. Each animal was placed individually into a new cage and brought to the observation room. Next, the animal was placed into the center of the dark testing chamber. The animal was given a 2 min adaptation period prior to testing. Following habituation, 10 min of testing took place. At the start of each minute, the light stimulus was presented for 3 sec. At the termination of the 10 min testing period, the animal was returned to the home cage for 60 to 90 min before perfusion. All behavioral procedures on testing day were video recorded for future observation and scoring. The frequency of freezing, exploring, self-grooming, and scratching behaviors and the duration of freezing, exploring, and self-grooming behaviors (Table 2) were recorded using The Observer (Noldus Information Technology; Wageningen, The Netherlands). It should be noted that duration of scratching behavior was not recorded due to the inability to accurately observe this measure.

Corticosterone Sample Procedures

Fecal samples were collected immediately before testing on day two of the behavioral procedures, approximately 24 hrs after the start of day one procedures. Three samples each weighing 0.1 g were collected from each animal and frozen unmixed in
sealed containers at -80°C until assaying. A total of 28 samples were collected and saved for corticosterone extraction and assay procedures.

*Corticosterone Extraction and Assay Procedures*

Prior to extraction, previously collected fecal samples were thawed at room temperature and placed in a glass tube with 1ml of 100% methanol. The contents of the tube were then mixed via the vortex machine for approximately 30 sec. Next, the tube was centrifuged for 10 min at 2500 rpm. Using a transfer pipette, the sample was transferred to a 13 x 100 mm glass test tube. The final step of extraction procedures was to dilute the sample in MeOH (concentration 1:20) in an EIA buffer.

Assay procedures were carried out using materials and protocols provided by an Enzyme ImmunoAssay (EIA) kit (Assay Designs, Anne Arbor, Michigan; Correlate-EIA, Corticosterone Enzyme Immunoassay Kit, catalog No. 900-097). The cross-reactivity of the kit was 100% with corticosterone, 21.3% with deoxycorticosterone, 21% with desoxycorticosterone, 0.46% with progesterone, 0.31% with testosterone, 0.28% with tetrahydrocorticosterone, 0.18% with aldosterone, 0.046% with cortisol, and less than 0.03% with pregnenolone, β-estradiol, cortisone, and 11-dehydrocorticosterone. The parameters of the EIA kit were determined and reported in a manual provided by Assay Designs. The assayed samples generated a line with a slope of 0.931 and a correlation coefficient of 0.999. Intra-assay precision (%CV) of the kit was 8%, 8.4%, and 6.6% for low, medium, and high concentrations of corticosterone, respectively. Inter-assay precision (%CV) was 13.1%, 8.2%, and 7.8% for low, medium, and high concentrations of corticosterone, respectively. Sensitivity of the kit was 26.99 pg/mL.
Sample readings were completed using an automated micro-plate reader (BIO-TEK, Richmond, VA, model # EL x 800) and the Kcjunior software (BIO-TEK, Richmond, VA, version 1.3, Part 5270501). Readings were assessed at a wavelength of 405\(\lambda\).

**Tissue Preparation**

Following the initiation of testing, the animals were killed and the brains removed and processed for neural analysis, 60 to 90 min after behavioral testing. Each animal killed with an overdose of pentobarbital sodium. Next, the animals were transcardially perfused with PBS, followed by chilled 4% paraformaldehyde (PF). The brains were postfixed in PF for three hours, followed by an immersion in 20% sucrose/phosphate buffered saline (PBS) solution. Brains were then blocked for the areas of interest (amygdala and hippocampus) and 40\(\mu\) sections were cut in the cryostat at -16\(^\circ\)C. The collected tissues were directly applied to sterile, subbed slides and frozen at 17\(^\circ\)C until immunohistochemistry (IHC) analysis.

**IHC Analysis**

\(C\)-\(fos\) IHC procedures were developed from protocols previously used and tested for reliability in our lab. All IHC procedures took place over four days. On Day 1, tissues were subsequently incubated in 5% Dimethyl sulfoxide (DMSO)/PBS solution for 10 min, incubated in .03% H202, 1% Normal Goat Serum (NGS) for 20 min, washed in five separate washes of 5 min durations in PBS, and stored over night in PBS at 4\(^\circ\)C. The following day (Day 2) the tissues were washed in two separate 5 min washes in PBS. Next, the slides were submerged in a blocking solution (PBS, 3% NGS, 25% Triton-X-100) for 2 hrs then the \(c\)-\(fos\) rabbit IgG primary antibody (Santa Cruz Biotechnology,
Inc., catalogue # sc-52, dilution 1:4000 [primary: blocking solution]) was applied to the tissues and stored in a humid chamber at 4° for 24 hr. On Day 3, the tissues were washed in six separate 10 min washes in PBS followed by a 2 hr incubation in a biotin conjugated, secondary antibody (Santa Cruz Biotechnologies, goat anti-rabbit secondary antibody, catalogue # sc-2040, dilution 1:500 [secondary: blocking solution]). Next, the tissues underwent three separate 10 min washes in PBS and a subsequent incubation in avidin-biotin enzyme reagent (ABC) solution (Vector Laboratories, catalogue # PK-6100). Another set of PBS washes was then completed (two 10 min washes). DAB (Sigma-Aldrich Laboratories, 3,3’-Diaminobenzidine, catalogue #D8001) solution was then applied to each tissue for 10 min, followed by six 10 min washes in PBS. The tissues were then placed under an enclosed hood overnight. On Day 4, series of ethanol, xylene, and deionized water washes were applied to the tissues prior to cover slipping. The schedule of washes is as follows: 50% ethanol for 2 min, 70% ethanol for 2 min, 95% ethanol for 2 min, 100% ethanol for 4 min, 100% ethanol for 4 min, 100% ethanol for 4 min, xylene for 4 min, deionized water until cover slipping. Lastly, permanent mounting medium (Polysciences Inc., Gold Standard Series/ Citra Mount Medium, catalogue # 24214) and coverslips (Gold Seal Cover Glass, product # 3246-000-900) were immediately applied to the tissues. Cover-slipped slides were stored at room temperature until observation by light microscopy.

c-fos Quantification

At the completion of all IHC procedures, the tissues were examined for condition and reliability of staining results. After much scrutiny and consideration, it was decided that too many remaining tissues were in unsatisfactory condition and also that the success
of the IHC procedures was questionable. Therefore, quantifying procedures were terminated and c-fos data was not collected.

Statistical Analyses

Prior to statistical analysis, it was determined that non-parametric analyses were most appropriate given that the assumption of normality was not met by the current dataset. Non-parametric Kruskal-Wallis tests were used to determine the effect of reproductive experience on the four dependent behavioral measures (freezing, exploring, self-grooming, and scratching) and corticosterone concentration. For significant findings, a post-hoc Mann-Whitney U test was employed to detect significance between individual groups (NP, PP, and MP). The nonparametric Spearman’s rho was used to in order to establish correlations between the behavioral (freezing and exploring duration) and hormonal measures within groups and also between the individual variables (freezing duration, exploring duration, self-grooming duration, and corticosterone concentration). The significance level of $P \leq .05$ was accepted for all tests.

A multi-dimensional scaling (MDS) analysis was conducted in order to provide a model of independent associations between the variables. The MDS constructed a visual representation or "map" of the distance (correlation) between the variables. The parameters of the MDS technique include the Kruskal stress index and the RSQ value. Goodness-of-fit, or how well the variables were accommodated by the dimensions of the model, was established by the Kruskal stress index. A stress value of 0.15 or lower was accepted as an indicator of good fit (Bardi et al., 2005; Manly, 1994). The RSQ is a percent value that determines the proportion of variance explained by the data set.
All statistical analyses were conducted using the SPSS computer program (SPSS 13.0, Chicago, IL.)

Results

*Behavioral Data*

A non-parametric Kruskal-Wallis test indicated significant differences between all reproductive groups (NP, PP, and MP) on duration measures of freezing [M=75.38, 30.15, 31.05 for NP, PP, and MP, respectively] (Figure 1) and exploring [M= 93.01, 133.65, 154.58 for NP, PP, and MP, respectively] (Figure 3) [freezing: H² =5.99, n =28, P =.05; exploring: H² =8.58, n =28, P=.014]. A post-hoc Mann-Whitney U test detected significant differences on freezing and exploration duration measures between NP and PP groups [freezing: U =19, P =.034; exploring: U =14, P =.011] and between NP and MP groups [freezing: U =20, P =.041; exploring: U =15, P =.014] with no significant difference between the PP and MP groups (Figures 1 and 3) (See Table 3 for means and P values).

No significant differences were detected between groups on all other behavioral measures: freezing frequency (Figure 2), exploring frequency (Figure 4), self-grooming frequency (Figure 5), and scratching frequency (Figure 7). (See Table 3 for means and P values). There was also no significance between all three groups on the self-grooming duration measure (Figure 6), but a Mann-Whitney U test detected a trend for the NP group to self-groom for longer durations than the PP group [U=23, P=.072].

*Corticosterone Data*

Prior to applying statistical analysis, the corticosterone concentration raw data set was examined for extreme scores. All scores below the kit’s sensitivity (26.99 pg/mL)
were replaced with the standard value (32 pg/mL). Using this guideline, two readings in the PP group and five in the MP group were replaced by the standard value. It was also determined that scores two standard deviations above the mean would also be discarded, eliminating one reading from the PP group. Following the above procedures, group means were obtained \([M = 792.58, 595.5, 92.05\) for NP, PP, and MP, respectively].

A nonparametric Kruskal-Wallis test was applied to the final data set indicating a significant difference between groups on the corticosterone concentration measure \([H_2 \neq 10.96, n = 27, P = .004;]\) (Figure 8). Post-hoc Mann-Whitney U analysis signified significantly different corticosterone concentrations between the NP and MP groups \([U = 4, P = .001]\). Though no significant difference was detected between the PP and MP groups, the Mann-Whitney U results indicate a trend for the PP females to have higher corticosterone levels than the MP group \([U = 17, P = .058]\). There was no significant difference between the NP and PP groups \([U = 29, P = .328]\).

**Spearman's Rho Correlation and Multi-Dimensional Scaling Results**

The nonparametric Spearman's Rho results indicated non-significance for the within group correlations between the behavioral measures (freezing and exploring durations) and corticosterone concentration. In reference to the correlations between individual variables across groups, the Spearman's rho detected significant correlations between exploring duration and freezing duration \((r = -.516, P = .005)\), exploring duration and self-grooming duration \((r = -.517, P = .005)\), and exploring duration and corticosterone concentration \((r = -.411, P = .003)\) (see Table 4 for all values).

The MDS technique generated Figures 9 and 10. The Kruskal stress formula determined a stress index value equal to .09, indicating a good fit between the dimensions.
Maternal Stress Response

and the mapped distances. The RSQ value designated that 96% of the variance was explained by the data. As represented in Figure 9, dimension one (x-axis) discriminated between exploring duration and freezing duration and thus was labeled the Fear dimension. Dimension two (y-axis) was labeled the Anxiety dimension because it discriminated between corticosterone concentration and self-grooming duration. Based on the two dimensions, the responses to the classical fear-conditioning model were divided into three major groups. The lower left quadrant represents animals that displayed long durations of freezing behavior and high corticosterone concentrations (Group 1). The second group is characterized by high durations of self-grooming and low corticosterone concentrations (Group 2). The final group exhibited long durations of exploring behavior and short durations of freezing behavior (Group 3). Based on the Kruskal Wallis and Mann-Whitney U results reported in the two previous sections, it was predicted that Group 1 represented the NP group, Group 2 the PP group, and Group 3 the MP group.

Figure 10 illustrates how the individual variables are mapped and, thus the distances between the variables. Variables 1-10, 11-18, and 19-27 represent the NP, PP, and MP scores, respectively. Given that the variables from each respective group are clustered together, Figure 10 confirms the previous assumption that Group 1 represented the NP animals, Group 2 the PP animals, and Group 3 the MP animals.

Discussion

The present findings provide new and considerable insights into the behavioral and biological modifications resulting from maternal experience. Measurement and analysis of the behavioral dependent measures detected significant differences between
the maternal and non-maternal groups in the expression of freezing and exploring behaviors within the designed model of learned fear. Additionally, corticosterone assays confirmed the prediction that female’s physiological stress response greatly decreases following reproductive experience. The differential behavioral and hormonal responses of maternal and non-maternal groups are reflective of the additional evolutionary investments and demands of motherhood. The correlation and MDS techniques elucidated several interesting relationships between the dependent variables and, thus provided supplementary information in support of the overall conclusion of this and other investigations in our lab: reproductive experience functions to shape the maternal mind and, as a consequence, secure reproductive survival.

The behavioral differences between the maternal and non-maternal groups elucidate how the maternal female manages to protect and care for her offspring in a threatening environment. Measurement of fear and anxiety-related behaviors expressed within a fear-conditioning paradigm are traditionally used to assess the extent to which the animal learns and remembers to fear a naturally unthreatening stimulus (Davis, 1992). The animal’s expression of fear-related behaviors, in this case freezing, when the CS is presented in the absence of the US, is an indicator that the association between the CS and US was learned and remembered. If the CS is continually presented without being followed by the US, then the animal is also challenged to learn that the two stimuli are no longer associated. In other words, it is expected that the animal will eventually habituate to the CS and refrain from engaging in freezing behavior. In evolutionary terms, both learning tasks of the fear-conditioning model are critical for survival, especially for that of the maternal female and her offspring.
Given that a higher incidence of freezing behavior was observed in the NP group than in both maternal groups, it is concluded that maternal experience facilitates and accelerates the learning and memory processes of the female. It is reasoned that because they were quicker to learn that the CS no longer predicted the US and, thus habituated faster than the non-maternal group. Investigations of other types of learning and memory tasks, such as unconditioned fear and spatial learning and memory, also report enhanced learning and memory capabilities, as measured by expressed behaviors, in maternal females (Kinsley et al., 1999; Wartella et al., 2003). Thus, it is evident that reproductive experience yields significant and ubiquitous improvements in the processes of learning and memory.

There is also evidence that the neural regions involved in the learning and memory processes are significantly modified by reproductive experience. For example, it is well understood that the hippocampus plays an integral role in the learning and memory of fear via its connections to the amygdala (Rudy et al., 2002) and, in addition, hippocampal function is necessary for the processing of spatial learning and memory (Rose, 2005). Interestingly, two primary pregnancy hormones, estrogen and progesterone specifically, induce increased growth of hippocampal dendritic spines (Woolley & McEwen, 1993). Investigations from our lab have confirmed this finding by demonstrating that administration of pregnancy hormones increases the density of hippocampal dendritic spines in non-pregnant rats (Kinsley & Lambert, 2006). Kinsley & Lambert (2006) explain that hippocampal modifications during pregnancy enhance the female’s spatial learning and memory skills, therefore contributing to her reproductive success. Considering the results of the current study, it is possible that the same
structural changes in the hippocampus are responsible for the female’s improved learning and memory abilities within a fear-conditioning model. In order to adequately care and protect her offspring, it is imperative for the maternal animal to recognize and remember that the CS no longer predicts the harmful stimulus. If the female remains fearful and freezes in response to the CS when there is no actual threat, she risks wasting energy and time that could otherwise be devoted to nursing, gathering food, and other critical maternal behaviors. It is therefore reasoned that neural modifications responsible for improvements in other forms of learning and memory are also associated with the observed enhancements in the fear learning and memory processes. Thus, the combination of past conclusions associating learning and memory with reproductive experience and the current findings affirm that the learning and memory processes necessary for survival are significantly improved following reproductive experience.

It should be noted that while the above explanation that maternal animals obtain enhanced fear learning and memory capabilities is certainly possible, the current study is limited in its ability to support this conclusion. Though the behavioral procedures were originally designed to measure the learning and memory of fear, in hindsight we are not confident that this goal was achieved. The reason for this uncertainty pertains to the introduction of novel toys on testing day of the behavioral procedures. It is possible though that the introduction of the toys on day two disrupted the classical conditioning model. Given that novelty functions as a source of mild stress for the rat (Kabbaj, Devine, Savage, & Akil, 2000), we are unable to determine if the differences in fear-related behavior between groups resulted from the presentation of the CS, the introduction of novel toys, or both. In order to ascertain that reproductive experience...
yields enhanced learning and memory skills within a classical model of learned fear, future research designs should exclude the introduction of novelty on testing day of the behavioral procedures.

In addition to the potential learning and memory explanation, differences in fear-related behavior between maternal and non-maternal females may be attributed to the maternal animal’s attenuated stress response. The Yerkes-Dodson law illustrates the effectiveness of an attenuated stress response in maternal animals, hence, while a degree of stress is critical for learning to occur, too much stress significantly impairs mental functioning (Ellison, 2005). Further, the animal is challenged to maintain a lessened stress response in a harmful or threatening situation in order to respond appropriately and survive. Considering her additional reproductive investments and responsibilities, an attenuated stress response is even more beneficial for the maternal animal. Past findings that maternal animals display fewer fear-related behaviors in an unconditioned model of fear and, in addition, less c-fos expression in the amygdala and hippocampus support the idea that an attenuated stress response is more critical for the maternal versus non-maternal animal (Wartella et al., 2003). Since the same relative pattern of freezing behavior across reproductive groups was observed in the present experiment, we are able to extend our understandings of the maternal attenuated stress response to a model of learned fear.

In addition to fear and anxiety-related behaviors, readiness to explore the surrounding environment is also considered an indicator of the animal’s current state of stress. As supported by the reported significant negative correlations between exploratory duration and the three dependent measurements of stress/fear observed
during the behavioral procedures (Table 4), exploration signifies that the animal was not experiencing high fear, anxiety, and stress levels. Here and in other research investigations (Wartella et al. 2003; Love et al., 2005), the maternal animals engaged in exploratory behaviors to a greater degree than the reproductively inexperienced subjects. Kinsley & Lambert (2006) deduce that the attenuated stress response permits the maternal female to leave her pups in order to explore the environment and obtain essential resources even when conditions are not necessarily optimal. In addition to this explanation, the learned fear results suggest that the attenuated stress response is required for the animal to first learn that the environment is no longer dangerous and second be willing to venture into and explore the surround.

The observed differences in exploratory behavior between maternal and non-maternal animals also highlight the effect of reproductive experience on the female’s response to novelty. As previously discussed, the employed fear-conditioning model included the introduction of novel toys on the testing day. Though this part of the design is problematical in reference to the assessment of fear learning and memory, it functioned to accomplish two other important goals. First, inclusion of the toys on testing day ascertained that the training and testing chambers were distinctively different, therefore assuring that the animals were conditioned to fear the hawk cry (CS) specifically, instead of the actual conditioning chamber. Secondly, the toys supplied the element of novelty to the behavioral design, which allowed for the examination of the effect of reproductive experience on the exploration of novel objects within a fear-conditioning model.

Novelty seeking is implicated in the learning process and, specifically, familiarization with the surrounding environment (Wilkinson, Herrman, Palmatier, &
Bevins, 2006). As previously addressed, it is imperative for the animal to establish familiarity with the environment in order to recognize new stimuli and potential threats. In addition, the introduction of novel objects is mildly stressful for rats (Kabbaj et al., 2000) presumably because the objects are unfamiliar and may jeopardize the animal’s survival. The presence of the toys, therefore, possibly contributed to and increased the animals’ stress levels on the fear-conditioning testing day.

Several research investigations, including the present, report that maternal subjects participate in exploration of novel objects to a greater degree than the non-maternal controls (Wartella et al., 2003; Love et al., 2005). This finding further emphasizes the maternal animal’s critical need to be familiar with her environment. The current fear-conditioning model presented two different stress-provoking stimuli during the testing trials: the presentation of the CS and the introduction of novel toys. Participation in novelty seeking despite both stressors reiterates the maternal female’s need to gather information about novel stimuli, which is fostered by the maternal attenuated stress response.

In addition to the current behavioral data, the comparison of corticosterone concentrations in maternal and non-maternal subjects also supports the above evolutionary explanations. The brain reacts to stimuli via hormonal release and regulation, which in turn influences the animal’s behavioral response. As stated in Becker & Breedlove (2002), behavioral and hormonal changes signify that the brain has also undergone some type of change. Thus, the current hormonal analysis functions to provide a proximate explanation for the observed behavioral differences and also gives information concerning the neural changes associated with reproductive experience.
As expected and in congruence with the behavior data, the maternal subjects displayed a smaller hormonal stress response than the non-maternal groups within the fear-conditioning model. Via negative feedback, the HPA regulates the release of corticosterone in response to stressors (Becker & Breedlove, 2002). Thus, the differential physiological stress response of the maternal and non-maternal groups may be attributed to changes in the HPA or its connections to other neural regions as a result of reproductive experience. Perhaps, the female’s threshold for a physiological stress response is decreased by a structural or chemical change in the HPA. Significant differences in corticosterone concentration between the maternal and non-maternal groups ascertain that some type of neural change occurs following reproductive experience, yet the details are unclear. The previously discussed differences in c-fos expression in the amygdalar and hippocampal regions of maternal and non-maternal rats (Wartella et al., 2003) suggest that decreased activation of these regions in maternal animals leads to changes in the chemical messages sent via their projections to the HPA. Presumably, modifications in these projections produce the attenuated physiological stress response observed in maternal rats. The verification of this interpretation demands further investigation beyond the scope of this study, but the present dataset provides insight into effects of reproductive experience on the biological mechanisms of the female’s stress response.

A secondary aim of this experiment was to determine if the behavioral and hormonal responses to the fear-conditioning model would vary depending on the animal’s number of reproductive experiences. Fairbanks (1993) explains that primate mothers learn from their first maternal experience and actually fine-tune their care for
offspring during subsequent events. Comparisons of the responses of the PP and MP subjects within the current study were designed to examine the possibility that such fine-tuning of maternal behavior might also apply to rodent mothers. Though the results demonstrate that such differences exist between maternal animals (regardless of the actual number of deliveries) and those completely lacking reproductive experience, they fail to clearly differentiate between animals with one versus two reproductive experiences. Notably, the statistical analysis detected a trend for higher corticosterone levels in the PP versus MP group (Figure 3), therefore hinting that the female’s hormonal stress response is modified with each new reproductive experience. Further investigation using a larger sample size may establish significant differences between the PP and MP groups on the corticosterone dependent measure, but we are currently unable to confirm this conclusion.

It should be noted, though, that other research studies examining fear and anxiety-related and exploratory behaviors in other paradigms, specifically the open field, dry land maze, and plus maze, also failed to detect significant differences PP and MP groups (Wartella et al., 2003; Love et al., 2005). These findings suggest that one reproductive experience is efficient for creating the neural and behavioral modifications necessary for rodent female’s reproductive survival. This explanation is logical given that the female’s ability to provide protection and care for her offspring is equally imperative for the survival of each litter, including the first. A second plausible explanation is that the female’s brain and behavior are augmented with each additional reproductive experience, yet these changes were not detected by the current research design. The reported fine-tuning of primate maternal behavior is in reference to actions such as attending to and
initiating physical contact with the infant (Fairbanks, 1993). Therefore, it is possible that observation of rodent FMB, as opposed to the current behavioral measures, would yield similar differences between PP and MP rats.

Dawkins (1989) explains that natural selection favors genes in the interest of survival, and thus highlights the solitary goal of evolution: survival. Thus organisms are driven to produce the most effective physiological and behavioral responses in toward threats and dangers in order to secure survival. In reference to the current study, all three reproductive groups were exposed to the same dangers, yet the responses of the maternal and non-maternal animals were distinctively different; therefore elucidating differential survival strategies between the groups. By explicating the relative relationships between the measured dependent variables, the correlation and MDS results delineate between the different types of survival strategies.

In reference to the correlation analysis, significant negative correlations were detected between the duration of exploring behavior and the freezing duration, self-grooming duration, and corticosterone concentration measures. These results differentiate between two possible responses to the fear-conditioning model: engaging in long durations of exploring and also spending less time freezing and self-grooming and, in addition, maintaining low corticosterone levels or, in opposition, spending little time exploring the fear-conditioning chamber and also freezing and self-grooming for long durations and exhibiting higher corticosterone concentrations. In congruence with previously discussed results, these two possible behavioral repertoires match those of the maternal and non-maternal animals, respectively.
From an evolutionary perspective, the maternal and non-maternal female’s two separate behavioral repertoires highlight the survival strategy employed by each animal. Remaining motionless and drawing as little attention to oneself is likely an effective survival strategy for the non-maternal animal that is not responsible for protecting a nest of noticeable and vulnerable pups. If the maternal animal adopts this same strategy, then she risks her pups being detected and attacked by a predator or not gathering the resources necessary for their care. The maternal animal’s survival strategy of exploring and gaining pertinent information about the environment, being less fearful of potential threats, and maintaining an attenuated stress response allows her to ensure her own survival and, in addition, that of her offspring. Though the correlation results highlight two distinct survival strategies between the maternal and non-maternal groups, they do not differentiate between the two maternal groups. The separate behavioral repertoires of the three reproductive groups in the present study are explained in further detail via examination of the MDS results.

The MDS technique provided a visual representation of the independent associations between the dependent variables, therefore mapping the variables on two meaningful dimensions. Dimension one, labeled the fear dimension, reiterates the negative correlation between duration of exploring and freezing behaviors; therefore, when the animal exhibited freezing behavior for long durations she was not engaging in exploratory behavior for long time periods, and vice versa. Dimension two, labeled the anxiety dimension, defines the relationship between self-grooming behavior and the hormonal stress-response. Self-grooming behavior carried out during high stress or anxiety provoking situations functions as a coping mechanism (Maestripieri, Shino,
Maternal Stress Response

Aureli, & Troisi, 1992; Castles, Whiten, & Aureli, 1999). The MDS diagram (Figure 9) explains that animals engaging in self-grooming for long periods of time also sustained relatively low corticosterone concentrations. Thus, the anxiety dimension is particularly interesting because it validates the self-grooming behavior as a successful coping mechanism for stress. The primary focus of Pavlovian fear-conditioning research is to uncover the learning and memory mechanisms of conditioned fear, whereas discussion of the animal’s methods of coping with the stress induced by the model is relatively limited. The relationship between self-grooming behavior and corticosterone concentration, as emphasized by the anxiety dimension, functions to broaden and extend research understandings of the rodent’s behavioral and the hormonal responses to conditioned fear.

The second visual representation created by the MDS technique (Figure 10) reveals that the behavioral and hormonal responses of the three reproductive groups are separated in terms of the two dimensions. Derived Stimulus Configuration (Figure 10) illustrates the Euclidean distance between the subject’s scores on the dependent measures. The orientation of the axes is randomly assigned by the statistical program and, therefore does not convey any pertinent information. The grouping of and distance between the individual variables, though, is interesting because it distinguishes between the three reproductive groups based on the two dimensions.

Examination of Figures 9 and 10, in conjunction with the results from the previously discussed statistical results, provides descriptions of each reproductive group in terms of the behavioral and hormonal responses to the fear-conditioning model. Based on all of the statistical results, three key conclusions were established. First,
the NP subjects are represented in Figures 9 and 10 as the variables that exhibit long durations of freezing and high corticosterone concentrations (Group 1). Second, Group 2 represents the PP animals, which are characterized by long durations of self-grooming behavior and low corticosterone concentrations. Third, the MP group is represented by the variables, which are characterized by long durations of exploring and short durations of freezing (Group 3).

The respective representations of each reproductive group give insight into how reproductive experience affects the female’s survival strategy. Reproductively inexperienced females recognize the situation or stimulus as threatening and respond by freezing, presumably to remain unnoticed. Motherhood, though, demands that the animal not only recognize and avoid danger, but also protect and care for her offspring. Thus, it is thought that the PP animals engaged in self-grooming behavior as a coping mechanism and a method of maintaining the attenuated stress response necessary for the completion of her maternal duties. It is possible that as a result of a second maternal experience, the MP animal recognizes that the fear-conditioning testing chamber presents no actual threat to survival and, therefore, expressed less fear and anxiety-related behaviors and engaged in exploration. Considered together, the MDS descriptions of the three reproductive groups give insight into a possible progression of changes in the female’s behavior and the stress response as reproductive experience increases. Further investigation and research on the relationship between reproductive experience and mechanisms of conditioned fear and the stress response will function to clarify the above MDS results.
Additional studies should also include an examination of the neural regions that are implicated in the processing of conditioned fear. Based on past research, it is clear that significant differences between maternal and non-maternal rodents exist in numerous brain regions including the cortex, mPOA, hippocampus, and amygdala (see Diamond et al., 1971; Keyser et al., 2001; Wartella et al., 2003). Given that the amygdala and hippocampus are key structures implicated in learned fear (Huff & Rudy, 1998; McEchron et al., 1998), it was predicted differential c-fos expression in these regions across reproductive groups would clarify the effect of reproductive experience on the neural processing of learned fear. Unfortunately, procedural difficulties impeded the collection of neural data in the present study. This information is critical, though, because it elucidates the integral relationships between the brain and the animal's biological and behavioral processes; therefore, yielding a more complete understanding of how reproductive experience modifies the female’s behavioral and biological responses to learned fear. Future studies are challenged to accomplish the above goal in order to further clarify the methods through which evolution promotes reproductive fitness.

The overall conclusion that maternal and non-maternal animals display separate behavioral and hormonal responses within the current behavioral model contributes to our understanding of how reproductive experience shapes the maternal mind and, as a consequence, secures reproductive survival. Based on the behavioral and hormonal differences between the three reproductive groups in the present study, it is concluded that reproductive experience significantly modifies the female’s behavioral and hormonal repertoire in response to fear-, anxiety-, and stress-provoking stimuli. This information adds to previous research findings concerning the maternal female’s marked
improvements in learning and memory ability, attenuated stress-response, and survival strategies; thus, highlighting the critical role that neural and behavioral plasticity plays in the female’s ability to obtain reproductive survival. Future research investigations should aim to address the limitations of the current study and, therefore, provide a more thorough and clear understanding of the relationship between reproductive experience and learned fear.
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Table and Figure Captions

Table 1. Schedule of US-CS Presentations
Table 2. Definitions of Behavioral Measures
Table 3. Means and $P$ Values of Behavioral Measures
Table 4. Spearman’s rho Results for Correlations Between Dependent Variables

Figure 1. Freezing Duration and Parity Graph
Figure 2. Freezing Frequency and Parity Graph
Figure 3. Exploring Duration and Parity Graph
Figure 4. Exploring Frequency and Parity Graph
Figure 5. Self-Grooming Duration and Parity Graph
Figure 6. Self-Grooming Frequency and Parity Graph
Figure 7. Scratching Frequency and Parity Graph
Figure 8. Corticosterone Concentrations across Reproductive Groups
Figure 9. MDS: Graph of Fear and Anxiety Dimensions
Figure 10. MDS Derived Model: Euclidean Distances between Variables
### Table 1

#### Sequence of CS-US Presentations

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## Definitions of Behavioral Measures

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<thead>
<tr>
<th>Behavioral Measure</th>
<th>Definition</th>
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<tr>
<td>Freezing</td>
<td>suddenly becoming rigid or motionless</td>
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<tr>
<td>Exploring</td>
<td>information gathering necessary for the formation of spatial representation and object discrimination (touching and sniffing toys)</td>
</tr>
<tr>
<td>Scratching</td>
<td>rapid movements of hand or foot to rake or pick the skin of the body</td>
</tr>
<tr>
<td>Self-Grooming</td>
<td>complex strings of movements to clean and maintain the fur and skin of the body (wiping, licking, and strokes)</td>
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### Means and P values of Behavioral Measures

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<tr>
<th>Behavior</th>
<th>NP (means)</th>
<th>PP (means)</th>
<th>MP (means)</th>
<th>P values</th>
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<tr>
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### Spearman's rho Results for Correlations Between Dependent Variables

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<th>CORT</th>
<th>FD</th>
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<td>1.000</td>
<td>-.517(**</td>
<td>-.411(*)</td>
<td>-.516(**)</td>
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<td>.033</td>
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<td>.216</td>
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</table>

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
Figure 1

Freezing Duration and Parity

Reproductive Group

NP

PP

MP

Duration of Freezing (sec)

75.38

30.15

31.05
Exploring Duration and Parity

Figure 3

Reproductive Group
NP: 93.01
PP: 133.65
MP: 154.58

Duration of Exploration (sec)
Exploring Frequency and Parity

Figure 4

Reproductive Group

NP

17.3

PP

19.78

MP

21.67

Frequency of Exploring Behavior
Self-Grooming Duration and Parity

Figure 5

Reproductive Group

NP  PP  MP

Duration of Self-Grooming Behavior

85.96  40.01  36.84
Scratching Frequency and Parity

Figure 7

Reproductive Group

NP: 3

PP: 4.44

MP: 3.89
Corticosterone Concentrations Across Reproductive Groups

Figure 8

Reproductive Group: NP, PP, MP

Corticosterone Concentration (pg/mL):
- NP: 792.58
- PP: 595.5
- MP: 92.05

Object Points: Common Space

Dimension 1
Figure 9

Object Points

Common Space

Dimension 1

Dimension 2

ZSGD
ZFD
ZED
ZCort
Derived Stimulus Configuration

Euclidean distance model
Curriculum Vitae

Brandi Nicole Rima was born in Norfolk, VA in January of 1982. She completed her undergraduate studies and research at The College of William and Mary in Williamsburg, VA and received her Bachelors of Science degree in Psychology with a minor in Biology in May of 2004. After completing her Masters of Arts degree in General Psychology at the University of Richmond, she will be attending The George Washington University in Washington, D.C. in order to pursue her doctorate degree in Applied Social Psychology.