QUANTIFYING PLASMA CORTICOSTERONE LEVELS FOLLOWING A CAREGIVING PARADIGM OF EARLY-LIFE ADVERSITY

by

Lauren Reich

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Approved:
Tania L. Roth, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved:
Carlton R. Cooper, Ph.D.
Committee member from the Department of Biological Sciences

Approved:
Jeffery Rosen, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved:
Michael Chajes, Ph.D.
Chair of the University Committee on Student and Faculty Honors
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ABSTRACT

Stress is a common stimulus for both animals and humans. To process stressful stimuli, the hypothalamus-pituitary-adrenal (HPA) axis is crucial in the physiological cascade that produces glucocorticoid stress hormones: cortisol in humans and corticosterone in rodents. Glucocorticoids are essential for proper development, circadian rhythm, and behavior; however, overexpression of these hormones can result in damaging physiological and behavioral changes, often through the modification of transcription and the epigenome. Previous work in our lab has determined behavioral and epigenetic consequences in rodents exposed to our well-established seven-day caregiving behavioral paradigm of early-life adversity. External research has indicated a stress hyporesponsive period in infant rodents, where even in the presence of aversive and stressful stimuli, a pup’s HPA axis will not be activated. Here we test whether our aversive caregiving conditions evoke corticosterone production, as corticosterone could be involved in our previously discovered behavioral and epigenetic consequences of the caregiving paradigm. Results indicated no significant change in corticosterone levels based on treatment group or sex. These data are consistent with other work showing the lack of a corticosterone response to stressful and aversive stimuli during the stress hyporesponsive period, and suggests that our behavioral paradigm induces epigenetic, behavioral, and neurological changes without the influence of increased corticosterone.
Chapter 1
INTRODUCTION

1.1 Response to external stressors

1.1.1 Historical context

In the field of psychology, there has historically been a debate of “Nature vs Nurture”. Proponents of the nature camp argue that human behavior is dictated solely by DNA sequence and is determined since birth. On the contrary, proponents of the nurture camp believe the environment is the primary reason to describe how individuals act and behave. Evidence that emerged in the early 2000s has helped us see that a blend of the two underlies behavior (Abrahamson, Baker, & Caspi 2002; Belsky et al., 2003). Further, our genes are up- and down-regulated based on changes in the environment (Moore, 2015), as the human body is a complex system that must adapt and exist in less-than stagnant situations. This change of gene expression is modulated by several pathways and varies on the type of stimulus imposed. Often, these transcriptional changes are dictated by epigenetics, where the genome is modified to have a change in gene expression to better suit the cell’s needs. In regard to tissue-wide modifications, these can influence physical health, mental health, and behavior (Ehrlich 2002; Casavant, 2019; Hamilton et al., 2019). These modifications vary in genomic location, chemical modification, and consequence, showing the remarkable adaptability of a living system.
1.1.2 Stress and biological outcomes

A stressful event is known to affect an individual’s wellbeing. In particular, early-life stressful experiences are widely known to contribute to negative outcomes in adulthood, often by increasing the likelihood of disease and/or risk-taking behavior (Nettis, Pariante, & Mondelli, 2019; Nieves et al., 2019; Lähdepuro et al., 2019). Early-life stress has also been linked to a higher likelihood of disease-related death in a dose-dependent association; a higher frequency of early-life hinted at a greater risk for mortal diseases such as cancer and cardiovascular disease (Russ et al., 2012).

Often, the caretaker is a common donor of early-life trauma due to the heightened dependence of a child to an adult. It is estimated that one out of every eight children experiences maltreatment from their caregiver (Wildeman et al., 2014). In altricial animals, offspring are heavily dependent on the caretaker in the early stages of life. It is known that these early interactions between caretaker and infant are crucial for neurological and behavioral development (Suomi, Harlow, & McKinney, 1972; Heim et al., 2009; Chambers, 2017). A fundamental study focusing on negative experiences related to caretaking environment is the Adverse Childhood Experiences (ACE) study. Using a questionnaire asking adult participants about their adverse experiences in their childhood household, researchers were able to link the frequency of early-life adverse experiences to mental, social, and physical deficits in adulthood. These deficits ranged from obesity, mood and anxiety disorders such as depression and posttraumatic stress disorder, and drug use (Felitti et al., 1998). Studies continue to provide support for early-life adversity as a predictor of negative adult outcomes (Anda et al., 2007; Brown et al., 2010). Due to the prevalence of these associations and the need to understand how these deficits manifest, it is crucial to better
understand how the body responds to developmental stressors, as this could allow us to create novel treatment strategies to combat these devastating outcomes.

1.2 The role of the HPA axis in stress response

Multiple regions of the nervous system are profoundly affected by developmental stressors (de Kloet et al., 1998; Doherty, Forster, & Roth, 2017). Of particular interest for this thesis study is the hypothalamus-pituitary-adrenal (HPA) axis; a stress-activated cascade that results in glucocorticoid release (Ulrich-Lai & Herman, 2009). When an organism is under stress, the hypothalamus is the first brain region stimulated. This tissue releases corticotrophin releasing hormone (CRH) and vasopressin into the medial eminence, where they can travel to various regions of the body. Together, the two hormones act on the anterior pituitary gland to stimulate the secretion of stored adrenocorticotropic hormone (ACTH). ACTH in turn is released into the bloodstream to stimulate the cortex of the adrenal glands. This adrenal activation initiates the synthesis and release of glucocorticoid hormones: cortisol in humans and corticosterone in most animal systems (Ulrich-Lai & Herman, 2009). When entering the bloodstream, these hormones are generally bound by glucocorticoid-binding globulin, which may facilitate the hormone’s entry into the cell (del Mar Romero et al., 2013). Once inside, the glucocorticoid can bind to an intracellular receptor and create a receptor-ligand complex that can interact with the promoter regions of many genes, thereby ultimately regulating transcription. These effects can take minutes to hours to unfold, however generally produce an effect on metabolic, immune, and cognitive function, generally acting catabolically (Sapolsky, Romero, & Munck, 2000; Sapolsky & Meaney, 1986). However, a receptor-ligand
complex working alone tends to repress transcription, while a dimer of two receptor-
ligands tends to activate transcription (Gray, et al., 2017).

Two receptors exist for these hormones: mineralocorticoid receptors and
glucocorticoid receptors. The first is restricted predominantly to the hippocampus,
while glucocorticoid receptors have a more widespread distribution in the brain (Reul
& de Kloet, 1985). Although the mineralocorticoid receptor has a higher affinity for
these stress hormones than the glucocorticoid receptor, the latter is thought to be the
true culprit of receiving and creating consequences of the stress response (Pariante &
Lightman, 2008). Due to the lower affinity of glucocorticoids to the glucocorticoid
receptor, the body will only undergo a stress response if a flood of glucocorticoids
enters the blood stream (Pariante & Lightman, 2008). If mineralocorticoid receptors
were the body’s detection system for the stress response, the system would likely be in
a hyper-responsive state due to its high affinity for glucocorticoids. For this reason, it
is widely understood that the glucocorticoid receptor is responsible for the somatic

The HPA axis operates on a negative-feedback loop, as increased
glucocorticoids bind to receptors located in both the pituitary and hypothalamus to
stop the potentiation of the stress-induced response (Ulrich-Lai & Herman, 2009).
However, in situations of prolonged stress, the stressful stimulus can override the
negative feedback loop and continue glucocorticoid production, thereby increasing
basal levels, as seen in those who have underwent trauma (Koss & Gunnar, 2018). Not
all incidences of HPA activation are negative however, as glucocorticoids are
necessary for brain maturation in regard to axon and dendrite remodeling, as well as
glucose utilization (Meyer, 1983). Not to mention, these glucocorticoids are essential
for the maintenance of circadian rhythms (Gray et al., 2017). Therefore, the HPA axis is crucial for proper physiological and neurological function.

1.3 **Relationship between glucocorticoids and epigenetic modifications**

1.3.1 **Epigenetics: an overview**

The link between developmental stress and negative outcomes in adulthood is often thought to be the work of epigenetic modifications in the brain (Vaiserman & Koliada, 2017). When a cell receives a chemical stimulus, temporary modifications in transcription can be induced via a second messenger system. However, for genes that need to be up- or down-regulated for a set amount of time, the cell primarily uses epigenetic modifications to accomplish this (Kanherkar, Bhatia-Dey, & Csoka, 2014). Genes that are not always constitutively active or inactive such as those involved in development, specialization, or homeostasis, are often epigenetically regulated (Mortada & Mortada, 2018; Matilainen, Quirós, & Auwerx, 2017). There are two common locations for these modifications: on the DNA itself, and on the amino acid tails of histone proteins, of which DNA wraps around (Moore, 2015). There are many different kinds of modifications that can be added to these locations, such as methylation, acetylation, and ubiquitination to name a few, however much is still unknown about their total effects on transcription (Moore, 2015). By investigating specific genes, experiments, and/or compounds, researchers are able to better understand how these modifications influence a living system.

1.3.2 **Glucocorticoid-mediated epigenetic modifications**

Corticosterone, a glucocorticoid, induces epigenetic modifications (Ewald et al., 2014). As glucocorticoids are known to induce a wide array of changes to
metabolism, development, and behavior, it is clear there must be many mechanisms by which these compounds behave. Ewald et al. (2014) focused on the ingestion of glucocorticoids and its significant increase in the methylation of a particular gene known to be sensitive to stress. Although a stressful stimulus was not given, the influx of stress hormone acted as a physiological stressor. This identifies a clear link between glucocorticoids and epigenetic modification, however much is still unknown about the full epigenetic capabilities of these stress hormones. Glucocorticoids also can act as a negative feedback loop to the HPA axis when they bind to receptors in the brain (Ulrich-Lai & Herman, 2009). As the brain is extremely intricate and influences many downstream processes, there is question as to what these bindings effect on a full-body level. Interestingly, the bound glucocorticoid receptor is known to have complex interactions with the genome that are still not understood (Hunter et al., 2014).

1.3.3 Manipulation of HPA axis activation

Regulation of the glucocorticoid response can also be mediated by regulating the genes critical for glucocorticoid detection and regulation of the HPA axis. Following a stressful event in early life, many studies have found significant changes on both the epigenetic modification and transcriptional levels of the gene coding for the glucocorticoid receptor (Weaver et al., 2004; McGowan et al., 2009; Perroud et al., 2011). By modifying the incidence of receptor formation, the cell has the ability to prevent or incite the HPA response, influencing many downstream processes that are associated with this protein. Studies show a link between increased DNA methylation, the decrease in glucocorticoid receptor transcription levels, and a history of adverse experiences in early-life (Weaver et al., 2004; McGowan et al., 2009; Perroud et al., 2011). As DNA methylation is known to suppress the transcription of a gene,
increased methylation of the glucocorticoid receptor gene suggests a decrease in the number of translated glucocorticoid receptors. This can therefore lead to a change in cellular response, as it decreases the number of receptors available for stress hormone detection. Another study highlights a microRNA localized in the brain that has been shown to decrease expression of the glucocorticoid receptor gene (Vreugdenhil et al., 2009). As seen here, the cell has multiple mechanisms to regulate the amount of glucocorticoid receptor being produced, thereby modifying the stress response which would have behavioral consequences.

1.3.4 Behavioral and developmental outcomes

The consequences of these modifications can potentially result in the change of behavior. This has been demonstrated in a study by McGowan et al., (2009), where suicide patients with a history of child abuse were found to have decreased gene expression of the hippocampal glucocorticoid receptor gene. As described earlier, multiple studies have found increased methylation of the gene responsible for glucocorticoid receptor formation in subjects exposed to early-life adversity (Weaver et al., 2004; McGowan et al., 2009; Perroud et al., 2011). Studies have also shown that a selective knockdown of glucocorticoid receptor expression in rodents is associated with increased HPA axis activity under both normal and stressful conditions, showing a hyperactive behavioral response regardless if a stressor is present (Ridder et al., 2005). Furthermore, mice with lower glucocorticoid receptor concentration were found to exhibit depressive-like behaviors (Ridder et al., 2005). This creates a connection between early-life adversity/stress, the physiological deficit of glucocorticoid receptors, and anxious or depressive tendencies.
De Kloet et al. (1998) further connects the influence of the HPA axis on the brain, showing that disturbances in mood, cognition, in behavior often correlate with abnormal levels of glucocorticoids themselves. In fact, the study shows that many patients who suffer from major depression have hyperactive HPA axis function, resulting in increased levels of glucocorticoids. Researchers believe the heightened HPA axis response is related to reduced feedback inhibition by endogenous glucocorticoids due to a lack of functioning glucocorticoid receptor (Pariante & Lightman, 2008). On the other hand, glucocorticoid receptor overexpression is associated with a lesser HPA axis response to acute stress (Reichardt et al., 2000). Both this increased reactivity and stress numbness can be detrimental in certain stressful situations. Although complex, a balance is critical for proper neurological function and survival.

Multiple human studies showcase the physiological effect of increased cortisol production. Researchers have found a link between elevated cortisol and reduced volume in the left hippocampus (de Kloet et al., 1998). Studies have found cortisol to influence the epigenome of specific age-related genes, which therefore cause a shrinkage of the brain region (Davis et al., 2017). Further studies on cortisol increase have also examined a decrease in the brain-derived neurotrophic factor gene, which is crucial for proper brain development (de Kloet et al., 1998; Smith et al., 1995; Li et al., 2019). This has also been implicated with other factors, such as the nerve growth factor, basal fibroblast growth factor, and transforming growth factor specifically in the hippocampus (de Kloet et al., 1998). It is clear that heightened cortisol has connections to brain and gene development, thereby influencing cognitive processing and future growth.
Early-life experiences are known to leave a long-term influence on the activity of the HPA axis. Rodents raised in varying nurturing conditions have a heightened response to further stress later in life. Champagne et al. (2003) shows the difference between rodent offspring exposed to high-frequency licking/nursing mothers and low nurturing mothers. Offspring with mothers who exhibited a low frequency of nurturing behaviors displayed a higher corticosterone response when in acute stress compared to their high nurtured peers. The low-nurturing dams may have acted as an early-life stressor or aversive stimulus, unable to provide for the pups when needed, thereby resulting in this HPA sensitivity. In humans, this heightened glucocorticoid response was also found in adult women who had undergone a serious trauma in childhood when later given a stressful stimulus (Heim et al., 2000). This shows the powerful influence of early-life experiences and their ability to alter stress reactivity later in life.

1.4 Stress hyporesponsive period

The influence of a caretaker is critical in the development of an infant (Greenberg, Speltz, & Deklyen, 1993). As humans can care for a crying child by nursing or rocking them, rodents often care for their young by licking and/or grooming them. While this promotes a bond and stimulates neuronal growth due to positive stimuli, this behavior also protects them against heightened corticosterone responses due to early-life stress (Weaver et al., 2004; Murgatroyd et al., 2015). Studies have demonstrated that the infant rodent is relatively unresponsive to stressors during the first few weeks of life if the mother is presenting nurturing behaviors (Loman & Gunnar, 2010). Specifically, immediately after birth, pups’ basal corticosterone levels begin to sharply decrease and remain at extremely low levels until approximately postnatal day 14 (Levine, 1994). Although following a different timeline, this same
phenomenon occurs in non-human primates and humans (Wiener et al., 1990; Loman & Gunnar, 2010).

This stress hyporesponsive period is suggested to protect the animal from major transcriptional changes while in a critical neurodevelopmental period (Lupien et al., 2009). Glucocorticoids have been found to be involved in the inhibition of cell division (Sapolsky & Meaney, 1986). In the developing rodent, this could create devastating consequences, as large spikes of growth are present in early-life. A delicate balance of glucocorticoids is needed, as both too high and too low levels of these hormones can interfere with development (Levine, 1994). Too high of levels can interfere with central nervous system development, however a low amount is essential; glucocorticoids are necessary for arousal, as it is involved with epinephrine secretion (Sapolsky & Meaney, 1986).

During the stress hyporesponsive period, the caretaker protects the subject from having a physiological response to stress. However, if the maternal bond is not formed, there is a lack of ability for the infant rat to gain this protective period. This can result in spikes in corticosterone response for the infant, even when subjected to low stress (Champagne et al., 2003). Without this nurturing behavior, the subject is more sensitive to stressors in the future, often creating a hyperactive somatic response (Loman & Gunnar, 2010). Human infants with an insecure attachment to their parental figure have difficulty moderating their cortisol spikes even when in the presence of a parent (Hertsgaard et al., 1995). This further shows the influence of a parental figure on an infant’s feelings of security, and its suggestions to irregular neurological development.
1.5 Maternal maltreatment as a form of early-life adversity

As mentioned previously, the interaction between mother and infant is known to have a large effect on the infant’s development (Greenberg, Speltz, & Deklyen, 1993) In our lab, we utilize a caregiving paradigm of early-life adversity to mimic maltreatment. By manipulating a rodent dam’s access to nesting resources in a novel environment while caring for infant pups, we can promote either negative or positive caregiving behaviors. Our well-established caregiving model of early-life adversity has been utilized for many experiments in our lab (Roth et al, 2009; Blaze et al., 2013; Doherty et al., 2017). Novel environments are known stressors for rodents, and by preventing habituation to this novel enclosure, we can produce a stressed or unstressed response. Furthermore, to properly care for young, rodents often build nests out of available materials, which in our case, is bedding material. By giving limited bedding, we can promote erratic caregiving behaviors. By manipulating these variables, we are able to provide an experimental setting to examine the effects of maternal maltreatment.

Multiple studies in our lab (Keller, Doherty, & Roth 2018; Doherty, et. al., 2017; Blaze & Roth 2017) have analyzed video and audio (both audible and ultrasonic) recordings taken during behavioral manipulations to determine if treatment groups are indeed receiving different caregiving behavior. For example, Keller, Doherty, & Roth (2018) demonstrated a significant difference in adverse and nurturing behaviors from dams assigned to the maltreatment group. This indicates that infants in the maltreated condition receive significantly more adverse and less nurturing behavior than their normal maternal care and cross-foster care counterparts (Figure 1).
Figure 1. Data recreated from Keller, Doherty, & Roth 2018 shows the distribution of adverse and nurturing behaviors during infant manipulations. The maltreatment group has significantly less nurturing and more adverse behaviors relative to subjects within the normal maternal care and cross-foster conditions. n=5 dams; error bars represent SEM; *** denotes p<0.0001.

Regarding pup vocalizations, Blaze & Roth (2017) measured vocalization frequency to determine if there was a difference between treatment conditions. As human infants cry out for help, rodents do the same, therefore giving researchers an ability to document a difference in experience and the caregiving behavior they receive (Portfors, 2007). Blaze & Roth concluded a significant increase in audible vocalizations in the maltreatment group when compared to the normal maternal care group. A marginal increase was measured in comparison to the cross-foster group (Figure 2A). For ultrasonic vocalizations, significant increase was found in the maltreatment condition when compared to both normal maternal care and cross-foster
care subjects (Figure 2B). This shows that pups in the maltreatment condition have increased vocalizations, thereby showing they receive more adverse care.

Figure 2. Data recreated from Blaze & Roth 2017. Shows the difference in pup vocalization across treatment groups. Pups exhibit significantly more (A) audible and (B) ultrasonic vocalizations in the maltreatment condition than their counterparts in the normal maternal care condition. n=7 litters; error bars represent SEM; * denotes p<0.05; # denotes p=0.079.
In regard to the infant pups, previous experiments have shown significant changes in brain development in multiple regions (Roth et al., 2009; Blaze & Roth 2017; Doherty, Forster, & Roth, 2016). These studies cite a variety of neural changes, such as alterations in gene expression via the manipulation of epigenetic markers. A conclusion of particular impact is from Roth et al. (2009), that discovered methylation of the brain-derived neurotrophic factor (Bdnf) gene following the caregiving paradigm. Crucial for proper brain development, the gene codes for a protein of the same name. This protein is responsible for promoting neuron growth, maturation, maintenance, and synaptic function (Charbonneau & Healy, 2005). The methylation of this gene provides insight onto how adverse caregiving behavior influences the brain. Studies from our lab have emphasized the behavioral outcomes of adult subjects that were exposed to our caregiving paradigm as infants (Doherty et al., 2017). From Doherty et al., (2007), results concluded an alteration in adult rodents’ cognition, memory, and depressive-like behaviors following the adverse caregiving paradigm. From this, it is clear that our caregiving paradigm has consequences that span the lifetime. Further, our work has showed some causality between the epigenetic modifications and behavioral outcomes (Keller, Doherty, & Roth, 2018).

1.6 The current study

Our caregiving paradigm has shown the effects of early-life maltreatment on the development of the rodent brain, as well as phenotypic outcomes later in life. Although from a conceptual and psychological point of view we utilize a behavioral model of stress, biological analysis on stress hormones has not been completed. As corticosterone has been found to be an epigenetic modifier, we are often asked as a laboratory about corticosterone levels following our paradigm. This is of particular
interest as the HPA axis is known to affect the epigenome and modify the transcription of various genes, such as our closely examined *Bdnf* (de Kloet et al., 1998; Smith et al., 1995; Li et al., 2019; Davis et al., 2017).

Thus, here we examine whether pups experience a corticosterone response following exposure to our caregiving paradigm, particularly the maltreatment condition. Based on the stress hyporesponsive period literature discussed earlier, we predicted we would not see a significant corticosterone response.
2.1 Subject generation

Long-Evans rats were bred in house with six total experimental litters produced from three dams. Breeder males and dams were only paired once to promote genetic diversity between our litters. Dams were bred initially with one litter produced before being used in experiments to control possible confounding variables associated with being a first-time mother. All animals were housed in 18 x 9 x 8in polypropylene cages, given ad libitum access to food and water, and maintained on a 12-hour light/dark cycle (lights on at 7:00 am). Day of birth was marked as postnatal day (PN) 0. On PN1, litters were culled to 12 pups when possible.

2.2 Caregiving behavior paradigm

The scarcity-adversity model of low nesting resources has been previously utilized by our lab (Roth, et al., 2009; Blaze, et al., 2013). Using a within litter design, pups were randomly divided into three caregiving groups: normal maternal care, cross-foster care, and maltreatment. For 30 minutes per day for seven days of early life (PN1 – PN7 or PN2 – PN8), pups underwent varying amounts of maternal caregiving or stress based on treatment condition. In the normal maternal care group, pups from the experimental group were weighed and immediately returned to their biological dam. In the cross-foster care group, pups were placed with an age- and diet-matched dam in a chamber (45.5 x 30.5 x 45cm Plexiglass chamber) with abundant nesting resources. Immediately prior to experimentation, the dam was given one hour to adequately habituate to her environment. Both the ample nesting resources and the habituation time are known to elicit nurturing maternal behaviors (Blaze, et al., 2013).
This is contrary to the maltreatment group, where pups and an unhabituated dam (also age- and diet-matched to the biological dam) were placed in a similar chamber with scarce nesting resources to promote erratic and aversive caregiving behavior (Blaze, et al., 2013). Following the 30-minute exposure, pups were placed back with their biological mother. Manipulations were performed at varying hours each manipulation day (during the light cycle) as to reduce any expectancy and to continue to promote a stressful environment for the maltreatment condition.

2.3 Biochemical analyses

On PN8 or PN9 (dependent on when manipulations began) after behavioral manipulations were completed, subjects were removed from the home cage, euthanized, and trunk blood was collected with 50µL of EDTA in order to prevent clotting. Collection was consistently performed in the afternoon to prevent time of day from being a confounding variable, as corticosterone levels follow a circadian rhythm (Halberg, 1969; Koss and Gunnar, 2018). Blood samples were spun down at 18,213 rcf for 10 minutes to segregate plasma from blood cells. A range of 20 to 600 µL of plasma were collected due to errors during blood collection. Once isolated, plasma samples were kept at -80 °C until further use. Corticosterone concentration was calculated by first generating a standard curve with provided standards (Corticosterone Enzyme Immunoassay Kit, Arbor Assays, Ann Arbor, MI). From this standard curve, unknown concentrations could then be determined for analysis. Duplicates of both standards and unknown samples were used to provide a more accurate reading.
2.4 **Statistical analyses**

Subject data was segregated by both treatment condition and sex. Two initial outliers due to extrapolation from the generated standard curve were excluded (MyAssays). Two remaining outliers within treatment condition and sex were also excluded (GraphPad Prism). If after the exclusion of outliers, a litter contained more than one pup in a particular treatment group and were of the same sex, their corticosterone values were averaged. This was done to avoid oversampling from a litter. A two-way ANOVA was used to decipher significance in sex and/or infant treatment condition.


Chapter 3

RESULTS

3.1 Corticosterone

For the current study, if a litter contained more than one pup of the same sex per treatment group, their corticosterone results were averaged to avoid over-sampling from within a litter (Holson & Pearce, 1992). A two-way ANOVA was used to assess corticosterone levels across treatments and sexes. As seen in Figure 3, there was no significance between treatment groups [F(2,27)=0.65, p=0.53] or sexes [F(1,27)=0.14, p=0.71]. In addition, there was no interaction between variables [F(2,27)=0.76, p=0.48].

Figure 3. Depicts the concentration of plasma corticosterone across treatment condition. No significance was found between treatment groups. n = 5-6 pups/group; error bars represent SEM.
3.2 Extraction day variability

Due to varying birthing times between the three treatment groups, three of the six total experimental litters generated were sacrificed on post-natal day 9 instead of the traditional post-natal day 8. To determine if this was a confounding variable, a two-way ANOVA was performed between infant condition and day of extraction. Values for both sexes were combined and averaged as there was no sex effect in the previous analysis. There was no main effect of treatment condition \( [F(2,27)=0.58, p=0.57] \) or day of extraction \( [F(1,27)=0.36, p=0.55] \). In addition, there was no interaction between these two variables \( [F(2,27)=2.29, p=0.12] \). Overall, this provides insight that the 24-hour difference between extraction times had no measurable consequence.
4.1 Caregiving paradigm successfully elicits treatment-dependent maternal behavior

The aim of this study was to determine if our caregiving paradigm was activating the HPA axis, the body’s primary response to a stressful stimulus. To do this, we measured pup plasma corticosterone levels following our seven-day caregiving paradigm. By measuring this stress hormone following our behavioral manipulations, we were able to assess the impact of our paradigm on HPA axis activity.

To definitively make conclusions about the consequences of our paradigm, we must first be certain that it truly manipulates caregiving behavior and that pups are responding differently to the treatments. Dams allocated to the maltreatment condition are placed in a completely novel environment with little to no bedding during the behavioral session. By manipulating these parameters, we promote erratic maternal behavior. As pups are subjected to these adverse behaviors, they vocalize, a measure suggesting their distress (Portfors, 2007). Due to time constraints, we were unable to analyze caregiving or vocalization behavior for the cohorts of animals used in this study. However based on previous observations summarized in Figures 1 and 2, we are confident that our paradigm provides adversity in the context of caregiving.

4.2 The stress hyporesponsive period protects our subjects from our stress-inducing behavioral paradigm

As a paradigm with a length of only 30 minutes per day for the first seven days of life, our paradigm could be considered only a mild stressor. Although mild, these
experiences are powerful enough to cause long-term epigenetic and behavioral changes (Doherty et al., 2017; Roth, et al., 2009; Doherty, Forster, & Roth, 2015). It is logical to assume that our maltreatment condition would activate the HPA axis, thereby producing a significant difference in glucocorticoid concentrations in maltreatment-group subjects. Our study however found no significant main effects of either treatment group or of sex, nor any significant interactions between the two. These data are consistent with other work showing the lack of a corticosterone response to stressful and aversive stimuli for the first few weeks of early pup life (Loman & Gunnar, 2010). As the behavioral paradigm has been previously shown to induce maltreatment-specific deficits in behavior and changes to the epigenome, data suggest from the current study that stress-induced elevations in corticosterone are likely not responsible for these changes.

4.3 Limitations and future directions

This project had various limitations. While seeing no significant difference in corticosterone levels, this suggests our pups are protected from HPA axis activation. However, it would be wise to test our model at a later age (subjecting older pups to the same caregiving conditions), such as a few weeks after birth, when the stress hyporesponsive period no longer exists (Loman & Gunnar, 2010). This would allow us to determine that our paradigm is truly stress-inducing and that this stress hyporesponsive period exists for only a limited amount of time in early life.

The project was also limited by the lack of sample collection at other points in time. For example, if we measured corticosterone levels immediately following our caregiving paradigm, we would be able to see if there was any instant activation of the HPA axis. This could allow us to better understand the stress-hyporesponsive period in
regard to our paradigm, as well as obtain a better picture of how our paradigm interacts with the physiological stress response.

Future direction in this line of work could perhaps focus on better understanding our paradigm’s effect on the brain’s glucocorticoid receptors. Although in rodents two receptors exist for corticosterone, the glucocorticoid receptor has a more widespread distribution in the brain (Reul & de Kloet, 1985). As mentioned previously, multiple studies have found significant epigenomic and transcriptional changes in genes necessary for glucocorticoid receptor formation in subjects exposed to a stressful early-life event (Weaver et al., 2004; McGowan et al., 2009; Perroud et al., 2011). By using these studies as a comparison, we can create our own investigations in the effects of our paradigm. Initially, this project can begin by measuring gene expression of the glucocorticoid receptor, in various brain regions (Agba et al., 2017). Further, tissue-specific investigation of epigenetic modifications on the gene’s promoter and/or exons could provide insight as to how transcription is regulated. Lastly, as the receptor is a cytosolic protein, a western blot could be utilized to approximate receptor concentration. Together, all of these methods could provide a better insight as to how our paradigm influences our subjects by investigating different brain regions as well as different developmental periods.

4.4 Conclusion

The findings in this study bring us closer to understanding how early-life stress induces behavioral and neurological outcomes later in life. For our purposes, the lack of significance of corticosterone concentrations across treatment groups suggests our behavioral paradigm has long-lasting effects into adulthood not produced by stress-induced increases in corticosterone. By studying a model of early-life adversity, we
hope to be able to provide insight into the biological consequences of development stress, which can provide valuable information to potentially aid those in need.
REFERENCES


