

2012

Effects of Elevated Prenatal Progesterone on Postnatal Emotional Reactivity in Bobwhite Quail (*Colinus Virginianus*) Neonates

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DOI: 10.25148/etd.FI12072701

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Miami, Florida

EFFECTS OF ELEVATED PRENATAL PROGESTERONE ON EMOTIONAL
REACTIVITY IN BOBWHITE QUAIL (*COLINUS VIRGINIANUS*) NEONATES

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

PSYCHOLOGY

by

Joshua A. Herrington

2012

To: Dean Kenneth Furton
College of Arts and Sciences

This thesis, written by Joshua A. Herrington, and entitled Effects of Elevated Prenatal Progesterone on Emotional Reactivity in Bobwhite quail (*Colinus virginianus*) Neonates, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

Lidia Kos

Anthony Dick

Robert Lickliter, Major Professor

Date of Defense: July 2, 2012

The thesis of Joshua A. Herrington is approved.

Dean Kenneth Furton
College of Arts and Sciences

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ACKNOWLEDGMENTS

I wish to extend my appreciation for my committee members, the developmental psychobiology lab team, and my friends and colleagues for their continued support. Many thanks for the guidance of my major professor, Dr. Robert Lickliter, who inspired me to boldly step into the unknown and emerge with a deeper appreciation and understanding of developmental science.

ABSTRACT OF THE THESIS

ELEVATED PRENATAL PROGESTERONE MODERATES EMOTIONAL
REACTIVITY IN BOBWHITE QUAIL (*Colinus virginianus*) NEONATES

by

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Florida International University, 2012

Miami, Florida

Non-genetic maternal influences on prenatal development have a significant effect on the development of early life behavior. This study assessed the behavioral effect of elevated prenatal progesterone on postnatal emotional reactivity, or underlying fear and stress, in embryos of Northern bobwhite quail (*Colinus virginianus*). Test groups of progesterone treated eggs, vehicle treated, and no injection were exposed to three measures of emotional reactivity at 48 and 96 hours after hatch: an open field, an emergence test, and a tonic immobility test. Heightened levels of emotional reactivity in the tonic immobility emergence tasks, and decreased levels of emotional reactivity in the open field suggest that elevated prenatal progesterone modifies post natal emotional reactivity up to 96 hours of age.

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CHAPTER I.

Introduction

Recent research has uncovered evidence that extra-genetic maternal influences can play a key role in their offspring's phenotypic outcomes. In contrast to reductionist views that argue for the genetic predisposition of phenotypic traits, recent studies have supported a more dynamic top-down/bottom-up approach to the development of behavior (e.g., Francis & Meaney, 1999; Caldji, Diorio, & Meaney, 2000; Meaney et al., 2004; Cameron et al., 2008). The results of these studies suggest that maternal behavior such as the amount of licking and grooming of rat pups by the dam influences physiological and behavioral outcomes in the pups (Caldji, 2000; Meaney, 2004). For example, rat pups that received prolonged maternal licking in the first week of lactation developed typical fear responses and normal sexual behavior (Francis, Diorio, Liu, & Meaney, 1999). In contrast, rat pups that received decreased amounts of maternal licking and grooming demonstrated atypical behavioral patterns that included an increase in fear responses, and modified emotional reactivity (Francis, Campagne, Mar, & Meaney, 2003).

Meaney and his colleagues (Weaver et al., 2004) have also explored specific epigenetic mechanisms that produce altered DNA expression, and found evidence that maternal licking alters DNA methylation patterns. At the molecular level, DNA methylation acts as part of an epigenetic system that affects the process of heritability by allowing or disallowing the transcription of specific genes within and also across generations (Bird, 2002). In this way, early life

interactions between the rat pup and dam facilitate a complex chain of biological reorganization that includes both top down (behavioral to molecular) and bottom up (molecular to behavioral) systemic reorganization. Meaney's research also suggests that maternal behavior can change the structure of DNA chromatin (Weaver et al., 2004). Chromatin, the structure within DNA is packaged, acts as an intermediary between promoters and transcription factors at the molecular level, which can modify patterns of gene expression (Kadonaga, 1998; Kouzarides, 2007). There is also evidence that increased pup licking and grooming by rat mothers alters the offspring's epigenome at the glucocorticoid receptor gene promoter in the hippocampus, which strongly suggests that non-genetic maternal behaviors can alter hypothalamic-pituitary-adrenal (HPA) responses to stress (Weaver et al., 2004). Thus, Meaney and colleagues suggest offspring emotional phenotype is determined by a complex set of factors at multiple levels of organization, involving genes, hormones, glucocorticoid receptors, epigenomic state, and maternal behavior.

CHAPTER II.

Literature Review

Maternal influences on the behavioral phenotypes of their offspring include many other non-genetic factors that take place during prenatal development, such as patterns and amount of sensory stimulation and the release of hormones (Lickliter & Schneider 2006; Richard-Yris, Michel, & Bertin, 2005; Mostl, Spendier, & Kotrschal, 2001). Exposure of mammalian fetuses to hormones of maternal origin (e.g., androgens and glucocorticoids) is known to play an important role in growth, the development of cognitive capabilities, stress responses, and sensitivity to later diseases, as well as the development of social and emotional behavior (Breuner, 2008). Unlike mammals, avian species have only one opportunity to deposit androgens in the embryonic environment of their offspring, which is during oogenesis. Analysis of the yolk and albumin of several species of birds revealed that eggs contain substantial concentrations of androgens, as well as other hormones of maternal origin that are essential for development (Gil, 2003; Romanoff & Romanoff, 1949). There is evidence to suggest that these androgens play a significant role in the emergence of phenotypic outcomes. Manipulations of maternal hormone yolk concentrations within the normal physiological range have shown that androgens can affect chick growth, behavior, immune function, and morphology, with effects that can last long after hatching (Groothuis, Muller, von Engelhart, Carere, & Eising, 2005)

Presently, it is unclear whether the transfer of hormones from a hen to her eggs is a passive transfer, or if there are unknown compensatory or environmental

mechanisms that control the transfer of hormones (Gil, 2003). Recent experiments exposing bird embryos to maternal hormones, such as cortisol and testosterone, have found that the increased presence of these hormones may influence growth and behavioral factors following hatching. For example, Japanese quail hens that were given artificial injections of corticosterone laid eggs with higher levels of corticosterone in the yolk compared to controls (Hayward & Wingfield, 2004). In addition, quail chicks with experimentally elevated corticosterone levels displayed different physiological and behavioral changes (i.e., decreased birthweight and increased emotional reactivity) when compared to chicks from non-manipulated eggs (Hayward, Richardson, Grogan, & Wingfield, 2006).

A study with bobwhite quail embryos found that elevating yolk testosterone levels before incubation resulted in a modification of phenotype (Bertin, Richard-Yris, Mostl, & Lickliter, 2009). Birds that had elevated levels of prenatal testosterone were compared to two control groups; one that received a sham injection, and a group that did not receive any injection. When compared across groups, the mean duration in tonic immobility (a classic test for emotional reactivity in birds) was significantly higher in testosterone treated chicks when compared to sham and control chicks. Testosterone treated chicks also had a faster growth rate over the course of early development, and showed evidence of enhanced prenatal auditory learning when compared to control chicks. A separate study found that Japanese chicks took longer to start distress vocalizations and produced less total distress calls in an open field when exposed to pre-incubation

elevation of testosterone, compared to control chicks (Daisley, Bromundt, Mostl, & Kotrschal, 2004). It also took longer for testosterone treated birds to display tonic immobility. In the general sense, these studies suggest that birds treated prenatally with modified levels of hormones display modified behavioral as well as physiological outcomes following hatching. These physical and behavioral outcomes tend to vary by species, developmental age, and the type and frequency of hormone manipulation.

Research Purpose

The main objective of the research project is to determine if artificial elevation of progesterone in the pre-incubation period can modify emotional reactivity following hatching in a precocial avian species (bobwhite quail). A review of related literature has shown that the study was the first to explore if modified exposure to prenatal progesterone has an effect on the postnatal behavior of bobwhite quail chicks.

Research Aims

By manipulating yolk hormone levels prior to incubation, the project aimed to understand the influence of progesterone during prenatal and early postnatal life. Experimental control and manipulation of the uterine environment in mammalian subjects is extremely difficult to attain. As such, utilization of quail eggs allowed investigation of prenatal endocrine processes that would be hard to explore with a mammalian model. Ultimately, the main aim of the research is to learn more about the development of emotional reactivity by way of non-genetic

maternal effects. Bobwhite quail embryos and hatchlings were used because (a) they develop in ovo and are thus easily accessible for prenatal observation and manipulation, including yolk hormone levels, (b) they are highly precocial, allowing for behavioral observations immediately following hatching, (c) they develop rapidly, which allows assessments of developmental outcomes over a relatively short period of time, (d) fertilized eggs are readily available from a commercial supplier (Strickland), which allows the onset of development to be highly controlled in the laboratory and (e) aspects of their perceptual and cognitive development has been observed and described in some detail in previous studies from our lab, allowing a useful baseline for our experimental manipulations (see Lickliter, 2005 for a review).

The study assessed whether prenatal exposure to elevated levels of progesterone acts as a moderator for emotional reactivity in bobwhite quail (*Colinus virginianus*). Upon hatching, the effects of elevated emotional reactivity were measured by three behavioral tasks: (a) the tonic immobility task, which has been correlated with general emotional reactivity in precocial birds, a response associated with underlying fearfulness (Bertin, Richard-Yris, 2004; Bertin, Richard-Yris, Mostl, & Lickliter 2009; Jones, 1986, 1987); (b) an open field social separation test that served as an additional measure of emotional reactivity; and (c) an emergence task that tested the chick's willingness to explore a novel environment. Tests will take place at 48 and 96 hours of age, to explore differences in developmental age. A previous study in our lab demonstrated that elevated prenatal testosterone levels resulted in increased latency in the tonic

immobility task, further suggesting that hormones present in the environment of the developing embryo can affect postnatal emotional behavior (Bertin, Richard-Yris, Mostl, & Lickliter 2009). The aim was to determine if elevated progesterone levels introduced very early in the prenatal environment could likewise significantly affect emotional reactivity following hatching. My study sought to test the following hypothesis:

Hypothesis # 1: Bobwhite quail neonates receiving pre-incubation exposure to elevated progesterone display modified emotional reactivity in the tonic immobility task when compared to control birds.

Hypothesis #2: Bobwhite quail neonates that receive pre-incubation exposure to elevated progesterone also display modified emotional reactivity in the open field and emergence tasks when compared to control birds.

Hypothesis #3: Behaviors differ at 48 and 96 hours of testing in the open field and emergence tasks.

CHAPTER III.

General Methods

Subjects

Northern bobwhite quail (*Colinus virginianus*) were used as test subjects. Three experimental groups were designated prior to incubation. The first group consisted of 10 eggs injected with progesterone infused corn oil, the second group of 10 eggs that received the corn-oil vehicle only, and the third group of 10 eggs that received no injection. The procedure was repeated until a total of 75 chicks were successfully reared and tested in each experimental paradigm. To achieve adequate power for the parametric and non-parametric statistical analyses an N of 25-40 chicks per experimental condition is usually required. Typical hatch rates vary from 50-60 chicks per week, but only about half of these are appropriate (i.e., correct developmental age, hatch on time, healthy, etc.) to test. To control for possible between-batch biases, subjects are drawn for each condition from two or more weeks.

Chick rearing

Incubation and post-natal rearing followed the protocol of previous studies in our lab (e.g., Bertin, Lickliter, Richard-Yris, & Mostl, 2009; Harshaw & Lickliter, 2011). Fertilized, unincubated eggs were shipped from a commercial supplier (Strickland) and set in a custom-built incubator. Approximately 48 hours before hatch, subjects were transferred to a portable hatcher. Subjects from all 3 groups were placed in the same portable hatcher, and separated by cloth and wire dividers. Temperature in the incubator was 37.5 °C, and the relative humidity

was 75-80%. Newly hatched chicks were identified by a colored leg band and weighed at Day 1. Their mass was recorded daily with an electronic scale. Chicks were transferred to a sound proofed rearing room and placed in groups of 12-15 chicks of the same age to replicate normative brood conditions. In order to ensure standard rearing conditions, chicks from all three groups were housed together. Ambient air temperature was maintained at approximately 35°C, and food and water were available *ad libitum*. Testing took place at approximately 12:00 h at 24 hours and 96 hours following hatching.

CHAPTER IV.

Experiments

Egg Injections

Hormonal manipulations mirrored the protocol utilized in a previous study in this series (see Bertin, Richard-Yris, Mostl, & Lickliter 2009). Prior to incubation, some of the eggs received 890.26ng of progesterone infused in 20ul of corn oil (Sigma), which raised the concentrations two standard deviations above mean levels. Progesterone was delivered to the yolk using a 26 G1/2 sterile needle after a small hole had been made in the egg-shell above the air sac with a sterile needle. The dose of progesterone used to elevate yolk levels is well within the natural range found in this species, which was ascertained by Dr. Eric Mostl for Bertin et al. (2009). Following the injection, the injection holes were sealed by either surgical glue or surgical tape. Two control groups were also tested. One group received no injection; a second received an injection of the carrier only (20 µl corn oil). Eggs were then be incubated to hatch in our lab.

Open Field/Emergence Task

All subjects from each experimental group (elevated progesterone, oil, and control) were tested in the open field task and emergence task at 48 and 96 hours after hatching. Open field social isolation tests measure general emotional reactivity and fear response by way of recording the amount of time spent in the periphery vs. center of the arena (Satterlee & Marin, 2006). Individual chicks were placed in a large circular arena in a sound attenuated room. Positional data collected by Ethovision XT 8.0 tracking software determined if the chicks in each

experimental group were in the center or periphery of the arena. The periphery of the arena was defined by an area which measured 2cm away from the arena wall and extended around the entirety of the perimeter. If the chick exceeded 1 minute 30 seconds in a state of immobility in the first 2 minutes of the open field experiment they were categorized as a non-responder. A state of immobility was defined as no detectable movement or vocalizations. Subject weight was recorded at the completion of the open field task with their leg bands removed.

The hole-in-the-wall emergence task was conducted in the same open field arena. It was designed to test the quail chick's willingness to emerge from a secure location and explore a potentially harmful novel environment. Birds were placed in a 10 x 17 inch box with a removable guillotine door panel facing the open field. Individual chicks were placed inside the box with the door closed for a period of 1 minute. After the 1 minute adjustment period, the door was opened and the time it takes for the bird to exit the box were recorded. At 5 minutes, the test ended and a maximum score of 300 seconds was recorded.

Tonic Immobility Task

The tonic immobility (TI) task has been widely used by animal researchers as a measure of general emotional reactivity in fowl (Bertin, Richard-Yris, Mostl, & Lickliter 2009). In the procedure, chicks are placed on their backs inside of a crinoline sling for 10s while the experimenter presses lightly on their chest. Tonic latency is recorded if the chick remains immobile for >10s, and if they right themselves or attempt to move <10s after the start of the task, an induction

attempt is recorded. If tonic immobility is not induced after five induction attempts, the birds received a score of 0 on the tonic immobility task.

No attempt to stand after 5min caused the test to cease and a maximum score of 300s were recorded. Pilot testing has shown that chick's escape from retention or chicks that are accidentally dropped to the floor prior to the start of the tonic immobility task display a heightened state of arousal, which may affect the duration of time spent in tonic immobility. As such, birds that escape from retention prior to or during the tonic immobility task were removed from the testing group. Test weights were recorded after the tonic immobility test with chick's leg bands removed.

Statistical Analysis

To achieve adequate power for statistical analyses of the hypothesis, we determined using a power analysis that an N of 25-40 chicks per experimental condition was needed to detect moderate effect sizes. Typical hatch rates vary from 50-60 chicks per week, but only about half of these are appropriate (i.e., they have the correct developmental age, they hatch on time, are healthy, etc.) to test. To control for possible between-batch biases, subjects are drawn for each condition from two or more weeks.

There were four dependent variables: mass of the chick, time spent in tonic, latency to emerge from the box, and time spent in the periphery. For the first analysis of mass, in which there were four repeated measures of mass, the design was a 4 (Age: 0 hours vs. 24 vs. 48 hours vs. 96 hours) x 3 (Condition: No Injection vs. Oil Injection vs. Progesterone Injection) repeated measures design.

For all other variables, a different sample of chicks was used. Here we concentrated on the 48 and 96-hour age groups, and thus the design was a 2 (Age: 48 hours vs. 96 hours) x 3 (Condition: No Injection vs. Oil Injection vs. Progesterone Injection) between-subjects design. In cases where there were no violations of assumptions, parametric statistics were used. In cases where there were violations of assumptions, we took appropriate statistical steps to deal with these, which are detailed below.

Results

Growth and Weight

Mass of the chicks was analyzed by way of a 4 x 3 repeated measures ANOVA.

There was no indication of weight differences or interaction effects between progesterone and non-progesterone treated at hatch throughout the first 96 hours after hatch ($p > .05$).

Table 1: Chick Weights

Mean \pm SEM mass of progesterone treated, oil treated, and controls chicks Day 1 - Day 4

Mass	Groups		
	Progesterone	Oil controls	Controls
Day 1	7.08 \pm .08a	7.19 \pm .10a	7.15 \pm .09a
Day 2	6.62 \pm .07a	6.79 \pm .08a	6.78 \pm .08a
Day 3	6.94 \pm .15a	7.05 \pm .14a	7.13 \pm .11a
Day 4	7.51 \pm .20a	7.44 \pm .19a	7.77 \pm .14a

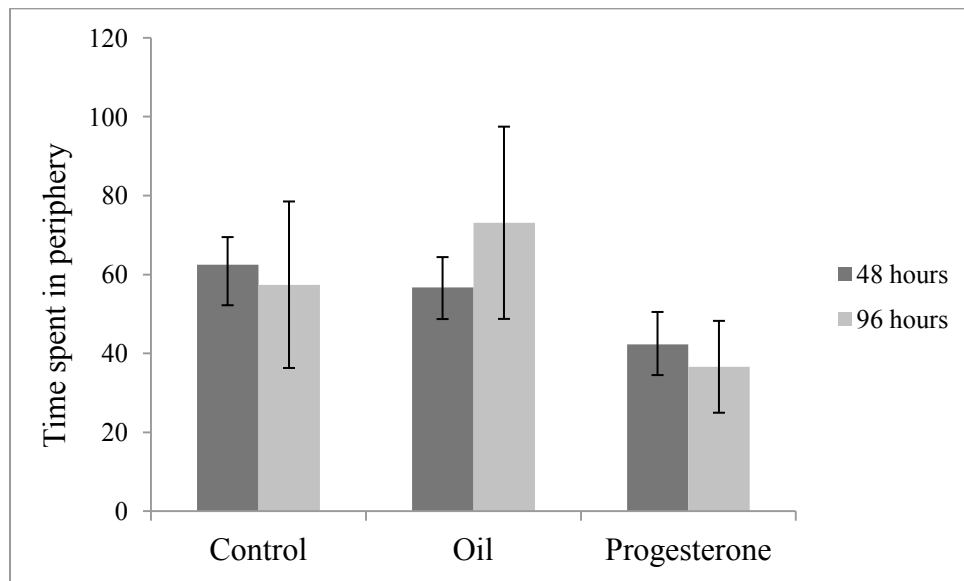
Different letters indicate significant differences in post-hoc HSD Tukey tests.

Open Field Test

Bartlett's test was completed to test the assumption of variance homogeneity between the 48 and 96 hour conditions. Results were non-significant $\chi^2(150) = 2.15, p = .14$, indicating no violation of the assumption of homogeneity. A robust

linear model was conducted, indicating no age ($t(148) = 0.19, p = .85$) or condition effects ($t(147) = -1.70, p = .09$), nor any interaction ($t(147) = -0.04, p = .97$).

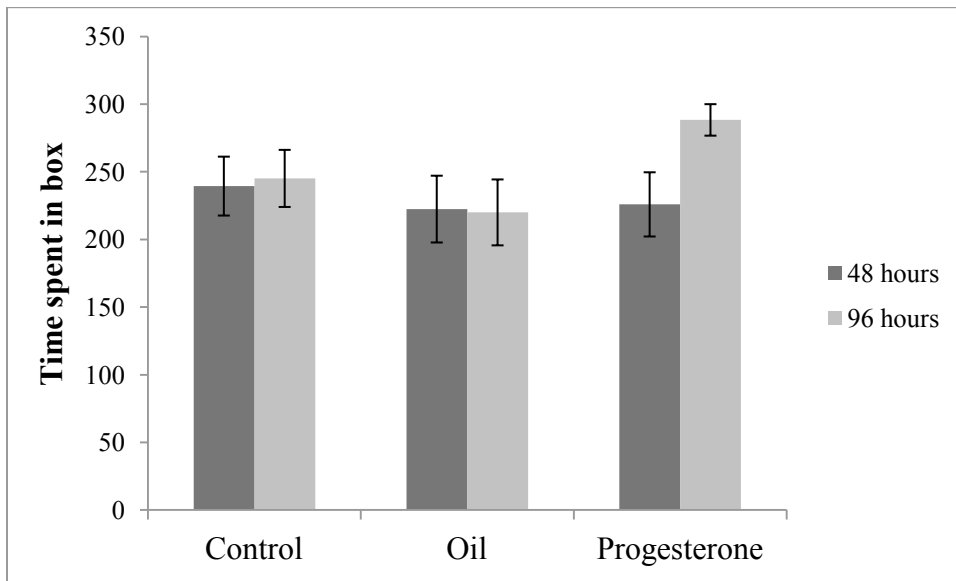
Figure 1: Mean Values of Open Field



Emergence Test

Birds tended to either stay in the box or emerge quickly. Therefore, I used a linear model with the assumption of a binomially distributed error. Condition differences were found to be significant ($t(146) = 1.97, p < 0.05$). In addition, there was a significant interaction effect age and condition ($t(146) = 1.97, p < 0.01$). Post-hoc tests determined differences in time spent in the box were significant between testing at 48 and 96 hours in the progesterone group ($t(46) = 2.01, p < 0.001$) and between the control and progesterone treated and oil treated groups. ($t(46) = 2.01, p < 0.001$).

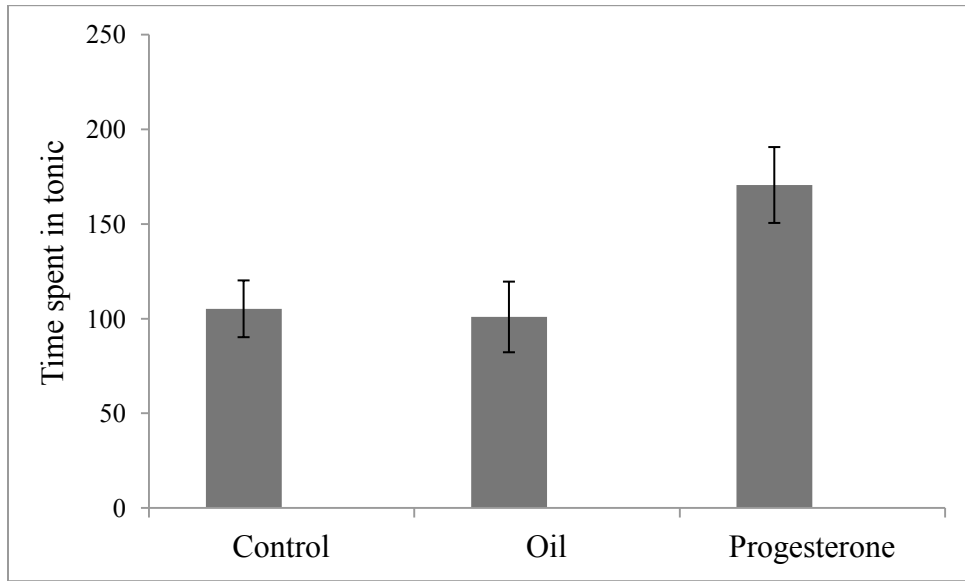
Figure 2: Means Values of Emergence



Tonic Immobility

As the tonic immobility data were found to violate parametric assumptions, Mann Whitney-U (Lehmann, 1975) tests were employed to determine differences in tonic immobility duration between groups. The omnibus test revealed significant differences in the latency score to exit tonic immobility (Kruskall-Wallace, $H(2) = 3.200, p = .005, N = 90$). Bonferroni corrected Mann-Whitney-U tests suggested that there was no significant difference in the duration of tonic immobility between the control and oil groups ($U = 411, r = -.07$), but that there was a significant difference between the progesterone and oil ($U = 280, r = -.32$) and the progesterone and control ($U=279, r = -.32$) groups. These data indicated that the progesterone treated birds spent significantly more time in a tonic state compared to the control and oil injected groups.

Figure 3: Duration of Tonic Immobility



CHAPTER V.

General Discussion

Discussion

Current research supports the theory that mothers transfer a great deal of information besides inherited genetic sequences over the course of development (for review see Harper, 2006; Lickliter, 2005). Non-genomic inheritance of emotional reactivity has been exemplified in both mammalian and avian species (Meany, 2004; Hayward, 2006). A study with Japanese quail found emotional reactivity was elevated in chicks that were reared by adoptive hens that exhibited high levels of emotional reactivity, and decreased in chicks reared by less emotionally reactive hens (Richard-Yris, 2005). The same modification of emotional reactivity can be obtained via direct manipulation of maternally derived hormones when yolk levels of testosterone are manipulated pre-incubation in bobwhite quail chicks (Bertin, 2009). Precocial species of birds are known to distribute maternally derived hormones in egg yolks differentially, dependent on lay order and the environmental conditions within which the mother is embedded (Gil, 2003). If cued from environmental conditions, these differences in maternal hormone distribution can offer information that can affect their progeny. For example, pregnant females who experience heightened levels of arousal and stress as a result of a stressful environment produce progeny that are highly reactive to stress (Richard-Yris, 2005). Experiments at the molecular and genomic levels in rats suggest a specific mechanism by which maternal behavior can influence fear and stress related behavior in their offspring. For example, impoverished amounts

of licking and grooming behaviors by rat dams alter the expression of glucocorticoid receptors in the hypothalamic-pituitary-adrenal axis of their offspring, which can influence their emotional reactivity (Weaver, 2004). Studies such as these explore specific mechanisms at several levels of organization (behavioral, genetic, and hormonal) and are crucial to the understanding of how emotional reactivity develops, is maintained, and how it can be modified.

It was suggested in a recent dissertation project that maternal distribution of progesterone in pre-incubated eggs is involved in post-natal behavioral modification in Japanese quail (Guibert, 2011). To the author's knowledge, the current study is the first to test the effects of elevated prenatal progesterone in bobwhite quail. The results of these series of experiments suggest that elevated progesterone modified time spent in tonic immobility, which met the assumption of the first hypothesis. In the tonic immobility test, progesterone injected birds stayed in a tonic state significantly longer than control groups. My results suggest that elevated prenatal progesterone results in the emergence of more emotional reactivity compared to the control groups. A similar result was found in a previous study in this series, where prenatal elevation of testosterone resulted in longer tonic immobility durations (Bertin, 2009). Contrary to similar experiments that involved prenatal cortisol and testosterone elevation in quail (Hayward, 2004; Love, Wynne-Edwards, Bond, & Williams, 2008; Bertin, 2009), no differences in weight or growth rate were found between the progesterone treated and non-treated chicks. Elevated pre-incubation testosterone has been found to increase

chick growth rates by 72 hours post-hatch (Bertin, 2009), while elevated prenatal corticosterone produced lighter chicks at hatch (Love, 2008).

When tested at 48 hours, there was no difference between groups in the time spent in the perimeter of the open field. Tests at 96 hours in the open field showed that progesterone treated chicks spent slightly less time in the perimeter of the open field, while the oil and control groups spent slightly more time in the perimeter of the open field. While there was a trend, there was no significant effect of progesterone on in the open field. For the emergence task, there seems to be a significant effect of progesterone on the emotionally reactive state of the bobwhite quail chicks. Tests at 48 hours show no difference in time spent inside the “hole in the wall” box, while progesterone treated chicks in the 96 hour emergence task took considerably longer to leave the box than control groups. Their behavior suggests progesterone treated chicks were less willing to leave the box to explore a novel environment, and therefore expressed higher levels of emotional reactivity compared to the two control groups.

Taken together, the outcome of these experiments present evidence that elevated prenatal exposure to progesterone has a modifying effect on postnatal emotional reactivity. It appears that elevated progesterone levels increase the propensity of emotionally reactive behavior in bobwhite quail neonates. Progesterone injected birds in the tonic immobility and emergence experiment displayed elevated emotional reactivity, while the results from the open field at 96 hours after hatch suggests that birds in an isolated, novel environment exhibited less emotional reactivity. It is possible that the tests of emotional reactivity used

in this age group (open field, tonic, and emergence task) may be measuring different fear and stress related responses, which has been previously suggested in a test-retest reliability study of fear related response in Japanese quail (Miller, Garner, & Mench, 2005).

Implications and Limitations

Demonstrative studies are an important first step to uncover the underlying mechanisms that drive the development of behavior. A thorough exploration of developmental mechanisms for any observable behavior should utilize a combined top-down and bottom-up approach. Presumably, there are multiple systems at the molecular, cellular, neural, and behavioral levels that interact dynamically in order to support differential outcomes in behavior; in this case, emotional reactivity. In this study, no such peripheral measures of stress outside of behavioral data that were collected. Baseline heart rate and peripheral corticosterone levels taken at baseline and post-test could help reveal the mechanisms that might underlie the modification of behavior in eggs with elevated progesterone. It is also unclear whether increased emotional reactivity via elevated prenatal progesterone extends into sexual maturity, or if there are any sex differences in the expression of fear and stress related responses.

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