Elevated Maternal Corticosterone Alters Offspring Development, Physiology and Behavior in Quail

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Abstract

Elevated Maternal Corticosterone Alters Offspring Development, Physiology and Behavior in Quail

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Chair of the Supervisory Committee:
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Within the last decade, a large body of literature has accumulated documenting the deposition of maternal androgens into egg yolk and their consequences for offspring. However, little is known about the transfer of maternal glucocorticoids into yolk and their effects on offspring. Here I show that high plasma corticosterone in a laying bird corresponds to high corticosterone in the yolk of her eggs. I then demonstrate that elevated yolk corticosterone has a range of effects on offspring development and adult phenotype.

Female quail from selected for heightened plasma corticosterone response to capture and restraint lay eggs with higher yolk corticosterone than quail selected for a low plasma corticosterone response. This difference in yolk corticosterone concentration is

particularly interesting given that baseline corticosterone levels do not differ between the two lines.

Experimentally elevating plasma corticosterone in laying quail increased corticosterone concentration in the yolk of their eggs. Chicks that hatched from eggs laid by females implanted with corticosterone grew more slowly than controls, and showed higher plasma corticosterone response to capture and restraint as adults.

To determine whether the effects of elevated maternal corticosterone were mediated by transfer of the steroid itself to yolk, yolk corticosterone concentration was manipulated directly. Male, but not female, chicks that hatched from eggs injected with corticosterone grew more slowly than controls, and female, but not male, offspring showed a decreased corticosterone response as adults.

Finally, eggs were injected with corticosterone and chick performance in a maze, and adult expression of anxiety behavior were quantified to investigate the effects of elevated yolk corticosterone on cognitive ability and behavior. Chicks from eggs injected with corticosterone completed the maze faster than controls on the first trial. However, control chicks improved in their second trial to become as fast at completing the maze as the corticosterone chicks. There was no effect of corticosterone treatment on anxiety behavior in adults.

Thus, I show that high maternal corticosterone is transferred to avian egg yolk and that it has lasting effects on offspring development and adult phenotype. Future studies are needed to understand the role of maternal corticosterone in yolk within an ecological context.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

PREFACE

When habitat parameters vary considerably over time, evolution will likely favor genotypes that code for different strategies under different environmental conditions. Whereas, a highly exploratory, aggressive coping strategy may be adaptive when predator density is low, for example, a more cautious, anxious coping strategy might be better suited to an environment with high predation pressure. Similarly, an animal expecting a relatively long life may postpone reproduction and invest in growth or in achieving social dominance, while an animal anticipating a relatively short life might invest earlier in reproduction. Because important parameters like food abundance and predator density vary considerably from year to year, different life history strategies may shift in relative adaptive value from one generation to the next. Thus, selection will favor an individual with the ability to adopt whichever strategy is best suited for the current conditions. In fact, most examples of highly disparate male reproductive strategies turn out to be phenotypically plastic.

Adopting the appropriate strategy for the current conditions involves responding to the appropriate cue or cues. Many life history strategies require a commitment to a single developmental trajectory early on, sometimes even before birth or hatch. In these cases, the cue or cues will most likely come from the mother, in the form of a maternal effect. Examples of adaptive perinatal programming orchestrated by maternal effects are abundant. For example, female insects that experience cooling conditions or decreasing day length often produce offspring that immediately enter diapause to survive the winter (Fox & Mousseau, 1998).

Not all maternal effects are adaptive, however. Some are merely constraints of physiology; the most obvious example being a mother in poor body condition giving birth to an offspring in similarly poor body condition.

Other maternal effects are more difficult to interpret. For example, in mammals, stress during pregnancy has been shown to have a range of effects on offspring, including increased anxiety, slowed growth, reduced learning ability, heightened sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis, and delayed puberty. Many of these effects, like slow growth and reduced learning, appear deleterious. However, it is possible that these costs are trade-offs associated with other traits that will tend to increase the offspring's fitness under the conditions of the local environment. The perinatal programming that occurs as a result of stress during pregnancy may be a constraint of physiology- the mother may need to increase her circulating glucocorticoids to cope with a stressor. She may not be able to effectively keep the high glucocorticoids from crossing into fetal circulation in the placenta, and the developing fetus may not be able to avoid suffering the debilitating consequences of exposure to high levels of maternal glucocorticoids. Alternately, high maternal glucocorticoids during pregnancy may signal to a developing fetus that local conditions are suboptimal. In response, the fetus may adopt a more reactive strategy, which will later help the fetus to maximize its fitness in the face of a challenging environment.

In many vertebrate species, a range of seemingly disparate traits are correlated into behavioral suites which have been described by Koolhaas et al. (1999) as "proactive" and "reactive" coping strategies. Proactive individuals exhibit high aggression, high exploratory tendencies, high routine formation, low anxiety and low sensitivity of the HPA axis. In contrast, reactive individuals are less aggressive, less

exploratory, less prone to routine formation, more anxious and show more sensitivity of the HPA axis; many of the same traits demonstrated by rodents exposed to prenatal stress. Koolhaas and his colleagues hypothesize that the proactive coping strategy is adaptive under stable conditions, whereas the reactive strategy may be an advantage when conditions are challenging. However, experimental evidence for their hypothesis is scarce.

I started my dissertation work interested in the potential for maternal stress to program offspring so as to maximize their fitness under suboptimal conditions. I had three goals. The first was to determine whether stress experienced by a laying bird would change the steroid content of her eggs. In particular, I was interested in deposition of corticosterone, the primary glucocorticoid of birds, into egg yolk. Second, I wanted to test whether high maternal corticosterone in egg yolk had lasting effects on avian offspring similar to those seen in mammals. Third, I wanted to explore whether the effects of high maternal corticosterone in yolk were adaptive for offspring or merely deleterious side effects of an adaptive stress response in the mother. The experiments presented here each use a different approach to test these three questions.

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I am lucky to have had the chance to work in John Wingfield's lab for the last six years. John has been unwaveringly supportive financially and personally through all the phases of my graduate career. I learned much from John's unique combination of talent, eminence and humility. Among other things, John is a phenomenal naturalist. I am grateful for having the opportunity to train with him in the field, both in Washington and in Alaska.

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DEDICATION

I dedicate this dissertation to my husband, Zachary Folk, who's support in its many forms has kept me sane from the time I thought I'd flubbed my interviews to the last days of anxiety about my defense. His involvement in my work has had him driving all over Washington and Idaho on a search for lazuli buntings; catching terns in the Columbia River; weighing Lapland longspur nestlings in Montana; photocopying grant proposals in the middle of the night at Kinko's; passing out paternity surveys at the SeaTac airport; hunting for redpoll nests in Barrow, Alaska, spending the night with hatching quail on the roof of Kincaid; weathering tropical storms in Florida; and collecting owl poop in Wenatchee, among other adventures. I am incredibly blessed to have such a partner. Thank you, Zac!

Chapter I: High Plasma Corticosterone Response to Restraint Correlates to High Yolk Corticosterone in Japanese Quail

INTRODUCTION

Elevated glucocorticoids in vertebrates function to bring the internal environment of an organism back to homeostasis after perturbation. Titers of glucocorticoids rise in response to disturbances like severe weather events (Wingfield, *et al.* 1983, Astheimer, *et al.* 1995), food shortage (Lynn, *et al.* 2003), and encounters with predators (Scheuerlein, *et al.* 2001, Boonstra, *et al.* 1998, Silverin 1998, Clinchy, *et al.* 2004). This generalized physiologic response to a stressor is referred to as the hypothalamic-pituitary adrenal (HPA) response to stress because glucocorticoids are produced by the adrenal in response to signals from the hypothalamus and pituitary. High levels of glucocorticoids act in the short term to redirect behavior and energy toward escaping and/or surviving a threat. To this end elevated glucocorticoids suppress reproductive, foraging and territorial behaviors, increase gluconeogenesis, and promote escape behavior and/or night restfulness (reviewed in Wingfield, *et al.* 1997, Wingfield, *et al.* 1998, Wingfield & Kitaysky, 2002).

Baseline levels of glucocorticoids may vary with body condition, social status and/or habitat quality and may indicate chronic stress in an individual (Wingfield, *et al.* 1998). In some cases, however, baseline levels of glucocorticoids are not affected by body condition or habitat quality while responsiveness of the HPA axis is (Bruener & Hahn, 2003). For example, Western fence lizards (*Sceloporus occidentalis*) infected with a malarial parasite have the same baseline levels of corticosterone as uninfected controls but show higher maximal levels of corticosterone in

response to capture and restraint (Dunlap & Schall 1995). Similarly, while there is no difference in basal corticosterone across the range of the Western fence lizard, maximal levels in response to capture are higher in lizards found at the periphery of their range than in lizards occupying more central habitat (Dunlap & Wingfield 1995).

In addition to the short-term, reversible effects of elevated plasma glucocorticoids discussed above (sometimes referred to as "activational effects"), elevated maternal glucocorticoids are known to have a broad spectrum of permanent "organizational effects" on developing offspring. For example, among rodents the effects of prenatal exposure to maternal stress include reduced learning ability (Weller, *et al.* 1988, Vallee, *et al.* 1997, 1999), heightened anxiety and activity of the HPA axis (Fride, *et al.* 1986, Henry, *et al.* 1994, Takahashi, *et al.* 1992a, b), reduced fertility (Herrenkohl 1979) and atypical sexual behavior (Ward 1972) (reviewed Herrenkohl 1986, Welberg & Seckl 2001). The effects of maternal stress may act in part to program a more "reactive" behavioral strategy (Koolhaas, *et al.* 1999), characterized by higher baseline anxiety and adrenocortical responsiveness to perturbation. Although difficult to test, Koolhaas and others (1999) speculate that a reactive phenotype may prove advantageous under challenging environmental conditions. Potentially, physiologic cues from the mother signaling poor local conditions may alter offspring physiology to optimize fitness in a poor quality environment.

Although embryos of egg-laying species are not directly exposed to their mothers' circulating steroids, as are mammalian embryos, they are exposed to maternal steroids in yolk (Schwabl 1993). Furthermore, these maternal yolk steroids are known to have organizational effects on offspring (Adkins-Regan, *et al.* 1995, Schwabl 1996b, Lipar & Ketterson 2000). For example, maternal cortisol in fish has been shown to reduce

the length and viability of larva (McCormick 1999) and reduce dispersal in common lizards, *Lacerta vivpara* (De Fraipont, *et al.* 2000). Also, elevated maternal corticosterone has been shown to slow chick growth and increase sensitivity of the HPA axis in Japanese quail (Hayward & Wingfield 2004).

Because egg constituents (albumen and yolk) are the only source of maternal steroids for young of egg-laying species, oviparous embryos are exposed to a more limited fraction of their mother's steroid profile than a mammalian embryo. Presumably, steroid concentrations in yolk correlate to plasma levels in the mother during only those few days while yolk is being formed by the liver and deposited, while placental embryos are exposed to circulating maternal steroids for the entirety of gestation and throughout nursing. While it has been shown that experimentally elevating corticosterone in laying Japanese quail increased the concentration of yolk corticosterone in their eggs (Hayward & Wingfield 2004), it is not clear how natural, transitory elevations of plasma corticosterone in a laying female relate to concentrations of corticosterone in her eggs.

We hypothesized that high plasma corticosterone response to non-specific stressors in Japanese quail would translate to high yolk corticosterone. In other words, we predicted that females more likely to experience exaggerated elevations in corticosterone in response to perturbation, would lay eggs with higher yolk corticosterone than females selected for reduced response. We tested our prediction by assaying yolk corticosterone in eggs from two lines of quail selected for either heightened or reduced plasma corticosterone response to brief mechanical restraint (high stress, HS or low stress, LS lines, respectively) (Satterlee & Johnson 1988). While these lines do not differ in their levels of baseline corticosterone (Jones, *et al.* 1994), they do differ significantly

in their release of blood corticosterone in response to a diversity of perturbations (Jones 1996). While quail were left relatively undisturbed prior to the first round of egg collection, they were exposed to the unavoidable perturbations of routine feeding and cage cleaning. In a follow-up study, we exposed both lines to controlled stressors (in addition to those stressors underlying the routine management procedures that were in common with the first study) and again assayed egg yolk for corticosterone to test the prediction that the anticipated difference between the lines could in this way be exaggerated. We also tested a secondary prediction that HS quail would lay eggs with lower yolk testosterone since high levels of corticosterone are known to suppress androgen production (Wingfield, *et al.* 1997).

METHODS

First Round of Egg Collection and Sampling:

When quail were 28 weeks of age, 24 eggs were collected from females of each line at Louisiana State University. Two eggs were collected from each of twelve family groups within each line on a single day (24 eggs from each line). Family groups consisted of ten females and five males housed communally in large breeding cages (one family per cage). Although exact maternal identity was unknown for each egg, collecting the eggs on a single day ensured that each egg was from a different female and could be treated as an independent sample. Whole eggs were shipped via overnight carrier to the University of Washington where they were prepared for assay within one week of laying. Yolk was sampled by cutting open the eggshell and manually separating the yolk from the albumen. Yolks separated from albumen were then weighed and homogenized with an equal weight of distilled water by vortexing in

a conical glass tube with several glass beads. Homogenized samples were frozen for later analyses.

Application of Stressors and Second Round of Egg Collection:

Prior to the second round of egg collection female quail of both lines were subjected to two stressor events. On day one of the experiment females were removed from their colony breeder (home) cages and cooped in their family group of 10 hens for two hours before replacement in their home cages (reinstatement with five home-cage, non-sibling resident males). This event involved stress for the quail associated with capture, handling and transport, both to the coop and back; separation from the familiar males of the home group and disruption of the social hierarchy; placement in a novel environment; and temporary (two hour) food and water shortage.

Three days later (on day four of the experiment) female quail were again removed from their home cages and family groups of ten were divided into two groups of five, each of which was placed in smaller pedigree cages with five unfamiliar members of another family group of the same line. Thus, in addition to the stressors of capture, handling, and transport to and from a novel environment, quail were exposed to unfamiliar conspecifics, overcrowded conditions, and transient isolation from familiar males. After four hours in the pedigree cages, females were returned to their home cages and familiar family groups. Three days later (day eight of the experiment) two eggs were collected from each family group (24 eggs from each line) and shipped to the University of Washington where yolks were sampled as described above. Although the family groups sampled were the same as in the first round of egg collection, the eggs may or may not have come from the same females. Therefore, samples were independent within round of egg collection but

not necessarily between rounds. Samples were taken from eggs laid seven days after application of the first stressor to ensure that the follicle had developed during the time that the female experienced the two stressors. Previous work has shown that elevations in plasma corticosterone translate to elevations in yolk corticosterone seven days later in Japanese quail (Hayward & Wingfield 2004).

Yolk steroid assay:

Samples from the first experiment (undisturbed birds) were run separately from samples from the second experiment (experimentally stressed birds). For the first assay 100 μl of each diluted sample (50 μl of yolk) was thawed, diluted with 400 μl distilled water and mixed with 20 μl tritiated corticosterone and 20 μl tritiated testosterone for calculation of recoveries. Samples were extracted twice with four mls diethyl ether and once with one ml 90% ethanol. Steroids were separated further by chromatography with columns of diatomaceous earth following a protocol developed by Wingfield and Farner (1975, modified Wingfield, *et al.* 1991), adapted by Schwabl for yolk (1993) and validated for this species (Hayward & Wingfield 2004). Because five samples were lost in the first assay when columns were blocked by yolk, only 80 μl of diluted sample (40 μl of yolk) were used in the second assay. Levels of yolk testosterone were measured in the first assay only (from eggs of undisturbed birds). Recoveries averaged 60% ± 8.99% (SD) for corticosterone and 54% ± 9.43% for testosterone in the first assay and 61% ± 5.47% for corticosterone in the second assay. Intra-assay variation was 9% and 7% for corticosterone and testosterone, respectively, in the first assay and 6% for corticosterone in the second assay.

Analysis:

Yolk hormone concentrations were compared between lines using a t-test for testosterone and a Mann-Whitney U-test for corticosterone because of heterogeneity of variance. Comparisons between experiments could not be made because eggs could not be considered independent. Nor were samples analyzed in the same assay.

RESULTS

Levels of yolk corticosterone were found to be about 62% higher in the HS line after the first round of collection wherein no stressors had been provided except those associated with routine management (average yolk concentrations were 2.9 ± 0.5 ng/ml for HS quail and 1.8 ± 0.2 ng/ml for LS quail; $U = 145.0$; $p = 0.04$; Fig. 1). After subjecting quail to the two experimental stressors, the HS line had yolk corticosterone concentrations 96% higher than the LS line (average yolk concentrations were 2.4 ± 0.3 ng/ml for HS and 1.2 ± 0.1 ng/ml for LS; $U = 84.5$; $p < 0.0001$; Fig. 1).

Average yolk testosterone did not vary between lines (4.6 ± 0.4 ng/ml for HS quail and 4.2 ± 0.3 ng/ml for LS quail; $t = 1.02$; $p = 0.31$).

DISCUSSION

By comparing two lines of Japanese quail selected for differences in plasma corticosterone response to restraint, we have shown for the first time that heightened adrenocortical responsiveness is associated with higher corticosterone in egg yolk. Levels of yolk corticosterone were around 62% higher in eggs laid by HS females when no stressors were purposely employed. This is not surprising, however, because, although

no stressors were applied intentionally to the hens during the week before egg collection, all quail were subjected to the stress associated with daily feeding and routine cage cleaning by student caretakers. We submit that the two selected lines perceived these daily stressors differently; that the stressors produced divergent plasma corticosterone responses; and thus, divergent yolk corticosterone concentrations. It is likely that HS birds experienced, on average, either more frequent transitory peaks of plasma corticosterone or peaks of higher magnitude in response to the events of their daily lives in captivity.

When all females were intentionally exposed to additional (experimental) stressors, the yolk corticosterone difference between lines was magnified to 96% (HS > LS). This magnified response may reflect the additive nature of the experimental stressors to those present during the practice of the routine husbandry procedures that were common to both experiments. We have focused on the line differences within round of egg collection rather than differences between round one (undisturbed quail) and two (experimentally stressed quail) because between-experiment comparisons would be less reliable given that different birds were likely sampled in each experiment, egg yolk samples were not necessarily independent, and samples were assayed for corticosterone in separate runs.

We found no support for our secondary prediction that yolk testosterone would be lower in the HS line of quail. Levels of testosterone did not differ between lines. This may reflect the fact that the expected transitory fluctuations of plasma corticosterone in the HS line do not have the effects of chronic stress in suppressing androgen production.

Collectively, the findings of both studies support the contention that free-living oviparous vertebrates with heightened HPA responsiveness due to poor body condition or habitat quality may

deposit relatively more corticosterone in their eggs. It is possible that the relatively high maternal corticosterone deposited in yolk by females with elevated HPA responsiveness may serve as a signal to the developing offspring of suboptimal conditions in the local environment and may cause changes to offspring phenotype that maximize fitness under challenging conditions. Preliminary work suggests that high maternal corticosterone causes high adrenocortical responsiveness in adult offspring (Hayward & Wingfield 2004, Hayward, *et al.* unpublished). Possibly, maternal corticosterone in yolk has organizational effects that serve to program a more reactive phenotype in developing vertebrates (Koolhaas, *et al.* 1999), better equipping them to cope with challenges such as high predator density or low food abundance.

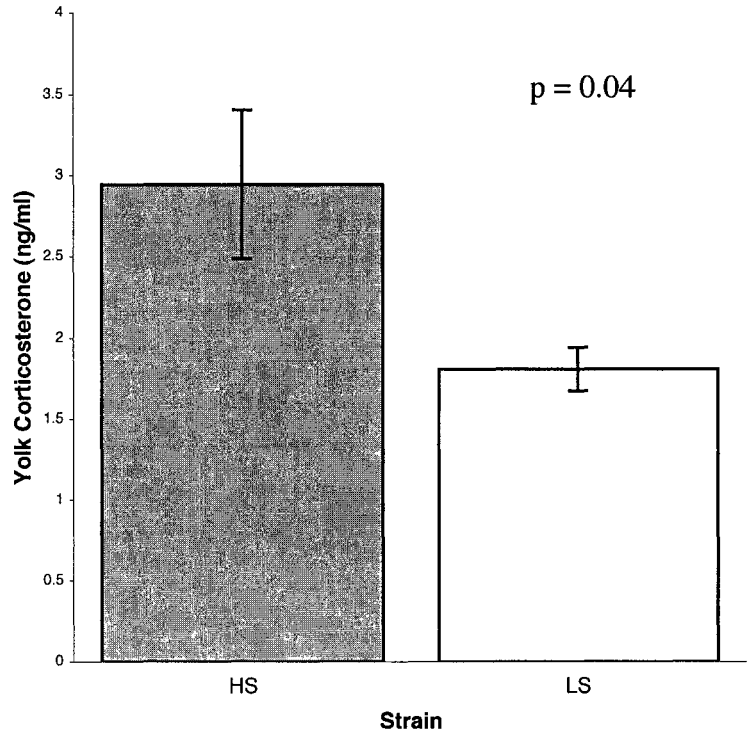
Alternatively, corticosterone levels may be high in yolk when they are high in the plasma of the laying female because of physiological constraints (Dufty, *et al.* 2002). There may be no mechanism by which a mother can keep corticosterone levels in her yolk from corresponding to corticosterone levels in her circulation. Similarly, there may be no way for developing embryos to protect themselves from the organizational effects effects of maternal glucocorticoids.

Although there is evidence that exposure to high yolk corticosterone may increase later HPA responsiveness in Japanese quail (Hayward & Wingfield 2004), it is not likely that the differences in adrenocortical responsiveness between the HS and LS lines are caused by differential deposition of corticosterone into yolk. Satterlee and Johnson (1988) showed an asymmetrical line development when first selecting the HS and LS lines of quail that favored progress in selecting in the upward direction. They found both that more selection pressure was possible in selecting high responders than low responders, and that responsiveness was more heritable when selecting for heightened

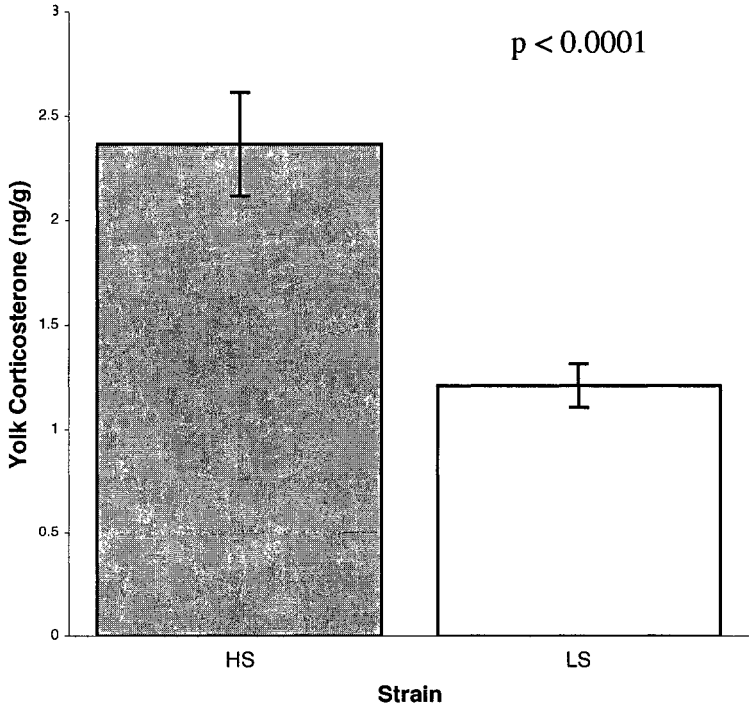
response. Recent analyses of the heritability of the stress responsiveness trait using diallel crosses supports Satterlee and Johnson's early findings by again showing that the trait has relatively higher heritability for selection in the upward direction and that the maternal effect for this trait is not any more significant than the sire effect (Odeh, *et al.* 2003a,b). Still, to a certain extent, there is potential for a positive feedback loop by which more responsive females lay eggs with higher yolk corticosterone and higher yolk corticosterone in turn results in adult offspring with higher adrenocortical responsiveness (Hayward & Wingfield 2004).

Figure 1: High Plasma Corticosterone Response Quail Lay Eggs With Higher Yolk Corticosterone Than Low Response Quail. Concentrations of yolk corticosterone vary significantly between quail selected for high or low stress response (HS and LS, respectively) when sampled after being housed normally (A) or after being experimentally stressed seven and three days before yolk sampling (B). Sample sizes were 22 HS and 21 LS in A and 24 HS and 24 LS in B.

A


p = 0.04
Yolk Corticosterone (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
HS
LS
Strain

B


p < 0.0001
Yolk Corticosterone (ng/g)
3
2.5
2
1.5
1
0.5
0
HS
LS
Strain

CHAPTER II: Maternal Corticosterone is Transferred to Avian Yolk and May Alter Offspring Growth and Adult Phenotype

INTRODUCTION

Many circumstances may elevate plasma glucocorticoids in vertebrates. Circulating levels of glucocorticoids in vertebrates often vary with environmental parameters such as weather, predator density or habitat quality. For example, snowshoe hares (*Lepus americanus*) have higher cortisol in times of high predator density (Boonstra, *et al.* 1998). The presence of predators in breeding territories has also been shown to increase plasma corticosterone in birds (Silverin 1998, Scheuerlein, *et al.* 2001, Clinchy, *et al.* 2004). Fence lizards (*Sceloporus occidentalis)* at the perimeter of their ranges have a higher adrenocortical response to capture than lizards more central within their range (Dunlap & Wingfield 1995). Northern spotted owls (*Strix occidentalis caurina*) with territories close to logging roads have higher fecal corticosterone than owls with territories further from disturbance (Wasser, *et al.* 1997), and wolves (*Canis lupus*) and elk (*Cervus elephus*) have higher circulating glucocorticoids during times of heavy snowmobile use (Creel, *et al.* 2002). Additionally, low body condition, disease or parasites can cause elevated plasma corticosterone or magnified response to capture and restraint (Dunlap & Schall 1995, Hood, *et al.* 1998, Breuner & Hahn 2003).

When mammals experience elevations in glucocorticoids during pregnancy, their offspring are also exposed to these circulating steroids, and often show long-term and wide-ranging alterations in phenotype as a result. For example, maternal stress during pregnancy in rats has been

shown to feminize male offspring, (Ward 1972); decrease the fertility and fecundity of female offspring (Herrenkohl 1979); increase anxiety behaviors in adult offspring of both sexes (Fride, *et al.* 1986); reduce learning ability (Weller, *et al.* 1988, Vallee, *et al.* 1999); and increase response of the hypothalamic-pituitary adrenal axis (Henry, *et al.* 1994, Takahashi, *et al.* 1992a, b, reviewed in Herrenkohl 1986, Welberg & Seckl 2001). There is also evidence of detrimental effects of prenatal stress in humans (Barker 1995, Huttunen & Niskanen 1978, Laukaran & van den Burg, 1980, Meijer 1985, Niswander & Gordon 1972, Stot 1973, Ward 1991).

Among egg-laying vertebrates, embryos are exposed only to those maternal hormones deposited in the egg during the relatively short period when yolk is being produced. However the organizational effects of yolk steroids can be important to offspring growth and development. In the last decade much evidence has accumulated documenting the transfer of maternal sex steroids to yolk; the influence of maternal environment on yolk steroid deposition and the effects of maternal sex steroids on phenotypic development of offspring (Adkins-Regan, *et al.* 1995, Eising *et al.* 2001, French 2001, Lipar 2001, Lipar & Ketterson 2000, Petrie, *et al.* 2001, Schwabl 1993, 1996a & b, 1997, Sockman & Schwabl 2000, Strasser & Schwabl 2000, Reed & Vleck 2001, Wittingham & Schwabl 2002). Fitness benefits for offspring from eggs with high levels of androgens include larger hatching muscle mass (Lipar 2001, Lipar & Ketterson 2000), faster growth rates (Eising, *et al.* 2001, Schwabl 1996b), higher dominance (Schwabl 1993, Strasser & Schwabl 2000) and better survival (Strasser & Schwabl 2000). Yolk testosterone concentrations vary not only among species, but also, within a species, both among and within clutches (Schwabl 1993, Schwabl, 1997, Sockman & Schwabl 2000). Interestingly, yolk androgen concentrations also vary

depending on the conditions to which the laying female is exposed, suggesting influence of the environment on physiology of the mother and consequently on the development of her offspring (Schwabl 1996a, Schwabl, 1997, Wittingham & Schwabl 2002). Also, in fish, yolk cortisol has been associated with reduced length of larvae at hatching (McCormick 1999); increased proportion of abnormal larvae (Morgan, *et al.* 1999); and higher egg mortality (Pottinger & Carrick 2000).

So far, little is known about the transfer of corticosterone, to avian egg yolk or its effects on offspring development. Until now it has been measured only in passerine eggs and been found in very low or undetectable levels (Schwabl 1993). However, because corticosterone is lipid-soluble like testosterone, it is likely deposited in egg yolk similarly and may alter offspring phenotype so as to maximize fitness under suboptimal conditions. Although the literature suggests that exposure to maternal glucocorticoids during development has predominantly detrimental effects on offspring, it is possible that energetic trade-offs exist that make these effects advantageous overall in a natural context. For example, black-legged kittiwakes (*Rissa tridactyla*) treated with exogenous corticosterone at 14 days of age demonstrated impaired learning ability eight months after treatment, similar to prenatally stressed rats. However, these corticosterone-implanted chicks demonstrated more frequent and aggressive begging for food while still in the nest, suggesting a competitive advantage also associated with exposure to high corticosterone early in development (Kitaysky, *et al.* 2001, 2003).

Given what is known about the deposition of maternal androgens in avian yolk and what is known about the organizational effects of glucocorticoids in vertebrates, we hypothesize that corticosterone will be transferred to egg yolk in amounts that correspond to circulating levels

in the mother at the time of laying, and that high levels of corticosterone in yolk will modify offspring development and phenotype. First we tested the predictions that experimentally elevating corticosterone in a laying bird would elevate the level of corticosterone in her eggs. Next we tested the prediction that chicks from eggs with high corticosterone would grow more slowly than control chicks and have higher hypothalamic-pituitary- adrenal responses as adults. We based our second predictions both on evidence that quail from a high stress response strain grow more slowly than quail from a related low stress response strain (Jones, *et al.* 1992, Jones 1996) and on the effects of maternal stress on offspring anxiety and adrenal response in rodents (see above).

METHODS

Study Species:

Thirty-four pair of adult (about seven weeks of age) Japanese quail (*Coturnix japonica*) were purchased from a local breeder (Boyd's Quail in Pullman, Washington) and brought into the lab. Pairs were housed in Hoei cages (approximately 36 cm x 38 cm x 43 cm) in an environmental chamber on 16 hour days at 25° C. Quail were provided with De Young brand game bird laying crumble (Woodinville, Washington) and water *ad libitum.* Quail were acclimated to laboratory conditions for eight weeks prior to implantation. All procedures were conducted with approval from the University of Washington Institutional Animal Care and Use Committee.

Implantation:

After acclimating to the laboratory environment and beginning to lay regularly, females were implanted with corticosterone (B-implanted)

or control tubes. Treatments were assigned randomly and interspersed throughout the chamber room. Implant tubes consisted of 16mm pieces of silastic tubing (Dow Corning, Midland, Michigan, USA) filled with crystalline corticosterone (Sigma, St Louis, Missouri) or left empty (control) and sealed at both ends with 1 mm of silicone sealant. Prior to surgery, the skin on the left flank was anesthetized with topical Benzocaine, sterilized with betadine and plucked of feathers. Implants were inserted under the skin on the left flank through small incisions (3 mm) that were then sealed with Nexaband brand veterinary glue. Just prior to implantation, the silicone sealant was trimmed from one end of the implant tube, leaving it open to ensure adequate release (Wingfield & Silverin 1986).

Validation of Implant Efficacy:

A separate study was conducted to validate the efficacy of corticosterone implants on female quail. Female quail of the same strain and age were housed individually in Hoei cages in a chamber room with 16 hours of light per day. After becoming acclimated, nine females were implanted with corticosterone and nine females were implanted with empty tubes (controls) as described above. Small blood samples were taken 24 hours after implantation and four days after implantation. Blood samples were obtained from a small puncture made to the alar wing vein with a 26 gauge needle within three minutes of capture to ensure that plasma corticosterone levels reflected baseline (Wingfield 1994). About 60 μl of blood was collected from each bird, separated by centrifuge and frozen until hormone assay. Hormone assay was conducted as described below.

Egg Sampling and Incubation:

Eggs were collected every day for one week following implantation, weighed and labeled. Eggshells were sterilized with alcohol prior to sampling. Twenty-six gauge butterfly needles (Abbott Laboratories, Chicago, Illinois, USA) and a 5 ml syringe were used to withdraw yolk from the center of each egg (Schwabl 1993). Samples were weighed, diluted with distilled water and frozen for storage. The puncture holes in the eggshells were covered with OpSite transparent wound dressing (Smith & Nephew Medical Limited, Hull, England).

Once sampled, eggs laid on day seven, or eight when a female failed to lay an egg on day seven, were placed in one of two Lyon TX7 Auto-turn incubators and maintained at 38°C and 50% humidity while being turned hourly. Eggs were collected seven days after implantation after a preliminary study showed that implants increased yolk corticosterone in eggs laid one week after implantation. This is likely due to the fact that it takes a female five to six days to form an egg from the first layers of yolk to laying.

Treatments were interspersed between the two incubators at random. Three days before hatching eggs were transferred to a brooder that maintained the same environmental conditions without turning the eggs. Within the brooder, eggs were placed in a grid constructed of wire mesh and cardboard dividers so that the identity of the chicks could be established post-hatching. Selecting only eggs laid on day seven or eight after implantation reduced our sample sizes to nine (B) and ten (control) because of failure of some females to lay and failure of some eggs to hatch.

Chick-rearing and comparison of adult physiology:

After hatching, chicks were marked with colored leg bands and placed into cohorts comprised of five treated and five control birds

(some chicks in these cohorts were not independent, having come from the same mother and therefore were excluded from analysis). Cohorts were reared in large pens with ad libitum food (De Young's game bird starter crumble) and water. Mass measurements were taken every day within the same hour.

Once chicks reached sexual maturity at eight weeks of age we measured activity of the hypothalamic-pituitary-adrenal (HPA) axis by obtaining a stress series (Wingfield 1994). Small blood samples of about 60 μl were taken from the alar vein at three, five, ten and 30 minutes from entering the chamber where the quail were housed. Between blood draws quail were restrained in soft cotton bags with minimal stimulation. Concentrations of corticosterone were calculated using radioimmunoassay described below.

Yolk corticosterone assay:

In order to validate the specificity and accuracy of the yolk corticosterone assay, pooled yolk was stripped of endogenous hormones with a concentrated charcoal solution (10 mg/ml). Yolk was then spiked with 4000 pg of corticosterone and serially diluted to obtain samples of 30μl yolk with known amounts of hormone. Spiked samples were then extracted with diethyl ether twice and ethanol once following the protocol described by Schwabl (1993) with the difference that pure diethyl ether was used rather than a diethyl ether/ petroleum ether mix. Short microcolumns of diatomaceous earth and glycols were used to separate and purify steroids, and competitive binding radioimmunoassay was conducted following the protocol developed by Wingfield and Farner (1975) and modified by Wingfield *et al.* (1991). Results of the validation assay indicate that other yolk compounds were not interfering with the

binding of the antibody to the hormone and that corticosterone could be measured in yolk (Table 1).

To run the yolk hormone assay, 50 μl samples were thawed over ice, diluted with 500μl distilled water and homogenized with a vortex and glass beads. Blanks and standards were set up and treated identically to samples. Twenty μl of radiolabelled corticosterone and testosterone were added to each sample for calculation of recoveries. Each sample was extracted twice with diethyl ether and once with ethanol. Samples were then run through columns, and steroid concentrations were calculated via radioimmunoassay as described above.

Analysis:

A repeated measures ANOVA was used to compare plasma levels of corticosterone for the implant validation study. The number of eggs laid in the week after implantation, egg weights and hatchling weights were compared using two-way t-tests. Yolk corticosterone concentrations were compared between treatments for days five, seven and nine after implantation using Mann-Whitney U test and Bonferroni corrections for multiple comparisons. Growth rates were compared by calculating a slope of growth between days two and six by linear regression for each chick and comparing slopes with a t-test. This part of the growth curve was used for the regression because it is a steep, almost linear phase of growth where differences in growth rate were most pronounced. To compare stress response between treatments we calculated the area under the curve of plasma corticosterone over time for each bird and used a t-test on the areas. This integrated approach provided a comparison of both corticosterone increase and clearance over the 30 minutes of restraint (Bruener, *et al.* 1999).

RESULTS

Implant Validation Study:

Corticosterone implants successfully elevated plasma corticosterone levels relative to controls within 24 hours of implantation (Fig. 2.1). Average plasma corticosterone for B-implanted females 24 hours after implantation was 11.68 ng/ml ± 3.46 while plasma corticosterone in control birds was 1.28 ng/ml ± 0.08 ($F = 7.172$; $p = 0.02$ for treatment effect). Within four days of implantation there was no difference in plasma corticosterone between treatments although implant tubes removed ten days after implantation were still approximately two thirds full of crystalline corticosterone.

Effects of Implantation on Laying and Yolk:

Corticosterone- implanted females laid the same number eggs in the week after implantation as control-implanted females (5.58 eggs and 5.61 eggs respectively; $t = -0.062$; $p = 0.95$; Fig. 2.2). Furthermore, the eggs that B-implanted females laid a week after implantation were the same size as eggs laid by control females ($t = -0.114$; $p = 0.91$; Fig. 2.2). However, implanting females with corticosterone significantly increased corticosterone in yolk of eggs. Eggs laid five days after implantation showed no effect of treatment on yolk corticosterone (0.93 ± 0.12 ng /g yolk in eggs of B-implanted females and 0.92 ± 0.13 ng /g in control eggs; $U = 28.5$; $p = 0.53$). However, seven days after implantation corticosterone had increased significantly in the eggs of B-implanted females (2.06 ± 0.26 ng /g yolk in eggs of B-implanted females and 0.92 ± 0.16 ng /g in control eggs; $U = 6.0$; $p = 0.003$; Fig. 2.3). Nine days after implantation eggs laid by B-implanted females tended to have higher yolk corticosterone than controls but differences were not significant

(1.58 ± 0.35 ng /g yolk in eggs of B-implanted females and 0.97±0.18 ng /g yolk in control eggs; U = 8.0; p = 0.12).

Effects of maternal treatment on offspring size, growth, and adult stress response:

Although there was no effect of treatment on egg size, the hatchlings from mothers implanted with corticosterone tended to have less mass than the hatchlings from control mothers. There is a trend for the difference between egg mass and hatchling mass to be larger in chicks from B-implanted mothers ($t = 1.86$; $p = 0.08$). Chicks from B-implanted mothers also grew more slowly in the week after hatching ($t = 2.622$; $p = 0.02$; Fig. 2.4). However, differences in mass disappeared by adulthood. Finally, a difference in adult stress response was detected between treatments. In response to capture and restraint, adult offspring of B-implanted mothers have higher levels of plasma corticosterone during the 30 minutes of restraint than controls ($t = 2.5$; $p = 0.02$ calculated from area under the curve; Fig. 2.5).

DISCUSSION

Our results show for the first time that experimentally elevating plasma corticosterone in a laying bird results in elevated corticosterone in the yolk of her eggs. Application of exogenous corticosterone elevated plasma levels for no more than a few days, simulating the physiological response to a transitory perturbation. Whereas our B-implants elevated plasma corticosterone to 11.68 ± 3.46 ng B /ml 24 hours after implantation, other quail of this strain have titers of 10.28 ± 1.47 ng B /ml after 15 minutes of mechanical restraint (Hayward, unpublished). These "stress levels" of plasma corticosterone are similar to those

reported in response to restraint for other strains of Japanese quail (Satterlee & Johnson 1988, Jones, *et al.* 1997), confirming that the levels resulting from our implants were within the naturally-occurring physiologic range. The fact that plasma corticosterone dropped in the B-implanted females before implant removal could be related to an increase in the steroid clearance rate and/or a decrease in the amount of binding globulin in circulation. It is not unprecedented for corticosterone titers to drop after rising in response to implantation (Kitaysky, *et al.* 2003), but, to our knowledge, this phenomenon has not been investigated in depth. The level of disturbance simulated by our implantation was not enough to interrupt laying (both number of eggs laid and egg size were unaffected by treatment) yet it did have an effect on the steroid content of the yolk (Fig. 2.3).

Experimentally elevating plasma corticosterone in the mother significantly affected the growth rates of her offspring. Chicks from eggs laid by B-implanted females tended to hatch lighter and grew significantly more slowly than control chicks (Fig. 2.4). It is possible that reduced growth rates lessen the burden of parental care although this is not an obvious strategy for a precocial species. Alternately, reduced growth rates may provide an advantage to chicks in the case of food shortage.

An additional possibility is that reduced growth is related to heightened fearfulness or anxiety, a trait that might prove adaptive in an environment with high predator density. Growth rates in Japanese quail correlate negatively with fearfulness (Jones 1996, Jones, *et al.* 1997). Furthermore, prenatal stress in rats increases "fearfulness" as measured through behavior in open field tests (Vallee, *et al.* 1997, Fride, *et al.* 1986, Takahashi, *et al.* 1992a, Williams, *et al.* 1998, summarized in Welberg & Seckl 2001) and activity of the hypothalamic-adrenal axis in

response to capture and handling (Henry, *et al.* 1994, Takahashi, *et al.* 1992a, b). We observed a similar effect of elevated maternal corticosterone in quail. Specifically, adult offspring of B-implanted mothers showed heightened activity of the HPA axis in response to capture and restraint relative to controls (Fig. 2.5). This may imply that levels of anxiety are higher in birds from mothers with elevated corticosterone, a characteristic that could prove adaptive in a situation where maternal corticosterone is elevated due to high predator densities.

In contrast, it may be that the effects of elevated maternal corticosterone on offspring are simply deleterious side-effects of the important function that elevated circulating corticosterone serves in the mother. There may not be an effective mechanism for blocking corticosterone from getting into the egg.

In summary, our results show that experimentally elevating corticosterone in laying quail increases corticosterone in egg yolk and has significant effects on offspring growth and adult physiology. Although it seems likely that maternal corticosterone in egg yolk may serve as the mechanism by which the effects of maternal implant on offspring growth and physiology occur, it is possible that other maternal effects are at work. Another study will be conducted in which yolk corticosterone is manipulated directly to control for other factors. Only then will we be able to assess the role of maternal corticosterone on offspring development.

Table 1: Amounts of corticosterone measured by RIA in samples of yolk stripped of endogenous hormone and spiked with known amounts of purified corticosterone. Values of the measured hormone represent averages of two spiked samples each run in duplicate. 2000 pg corticosterone were added to the first sample and a serial dilution was performed with the stripped yolk to obtain the other hormone amounts.

Hormone added to yolk (pg)	Hormone measured in yolk (pg)
1000	1033.09
500	553.84
250	242.74
125	111.61
62.5	61.08
31.25	37.88
15.62	16.29
7.81	12.82
0	Non detectable

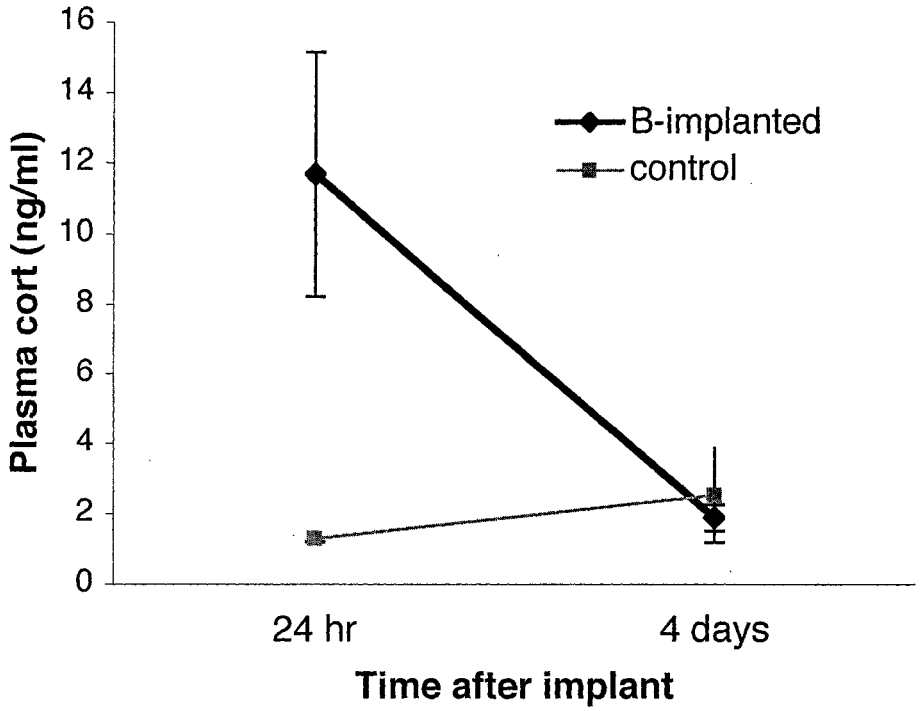


Figure 2.1: Corticosterone (Cort) implants significantly increased plasma Cort in female quail within 24 hours relative to control-implanted females (U = 8.0; Tied p = 0.004). However, at four days after implantation there was no difference in plasma Cort between treatments. Sample sizes were nine and nine.

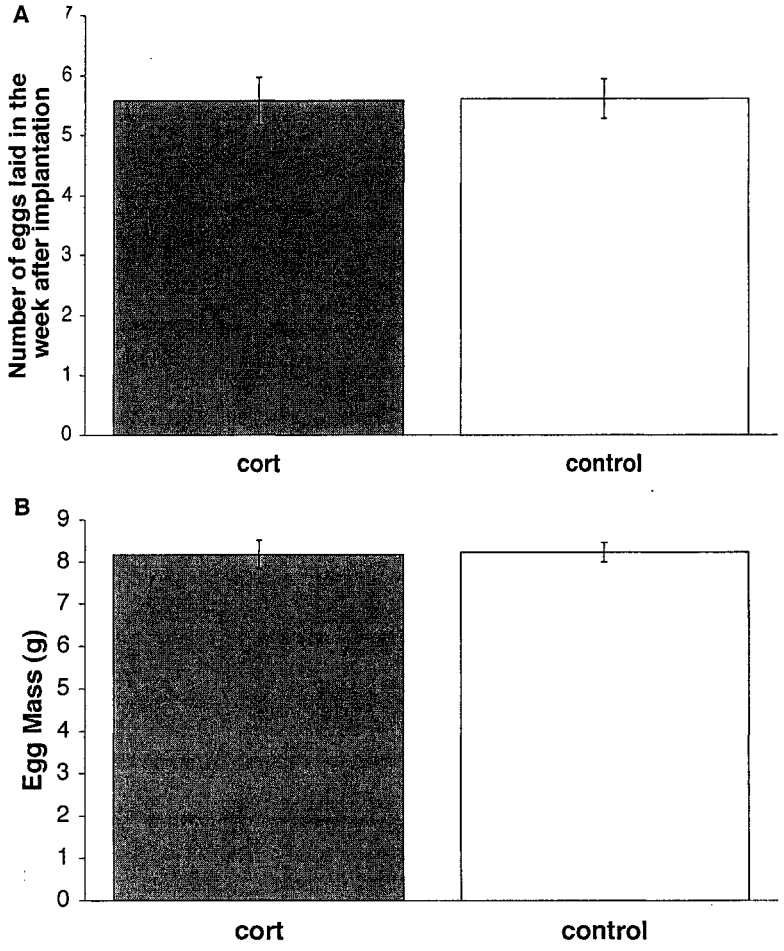


Figure 2.2: Corticosterone-implants did not affect egg production. There was no effect of treatment on the number of eggs laid in the week after implantation ($t = -0.062$; $p = 0.95$; A). Sample sizes were 12 corticosterone-implanted females and 13 controls. Neither was there an effect on the mass of eggs laid one week after implantation ($t = -0.114$; $p = 0.91$; B). Sample sizes were 8 eggs from corticosterone-implanted females and 10 eggs from controls.

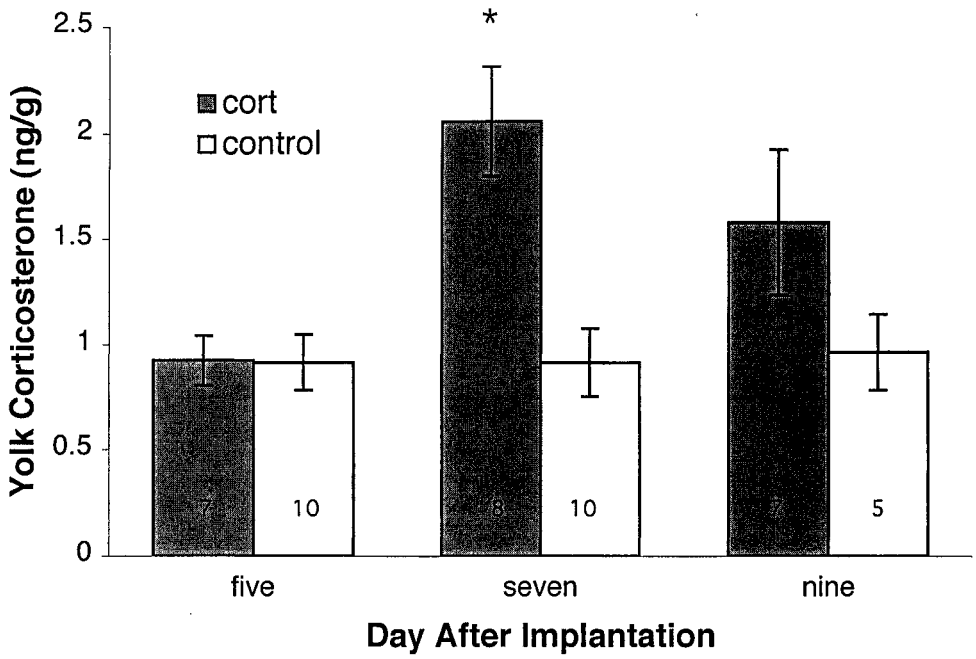


Figure 2.3: Corticosterone implants significantly increased the concentrations of corticosterone in yolk of eggs laid seven days after implantation (U = 6.0; p = 0.003). Five days after implantation there was no effect of treatment on yolk corticosterone (U = 28.5; p = 0.53) and nine days after implantation the eggs of corticosterone-implanted females tended to have more yolk corticosterone but differences were not significant (U = 8.0; p = 0.12). Sample sizes are shown.

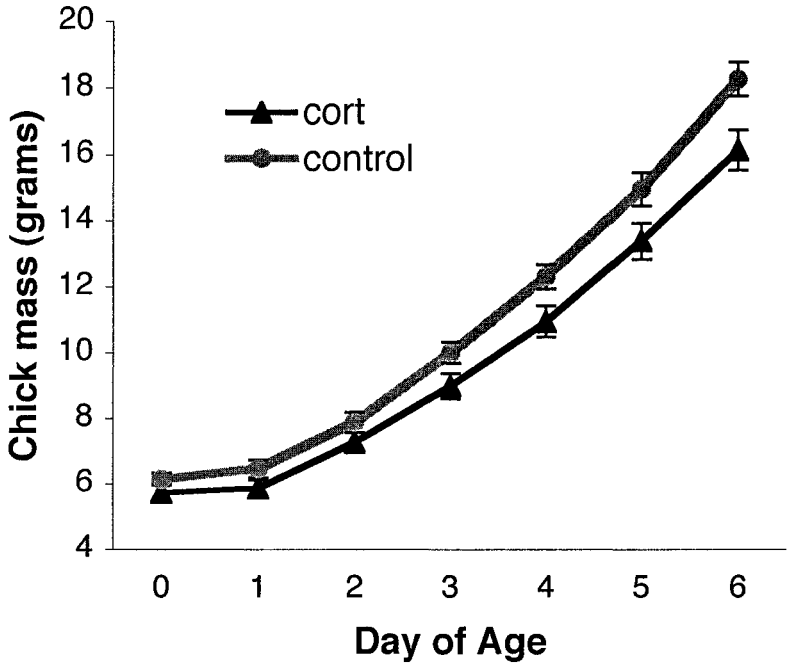


Figure 2.4: Chicks of mothers implanted with corticosterone grew more slowly than chicks of control mothers in their first week after hatching ($t = -2.622$; $p = 0.02$). Sample sizes were nine corticosterone and ten control.

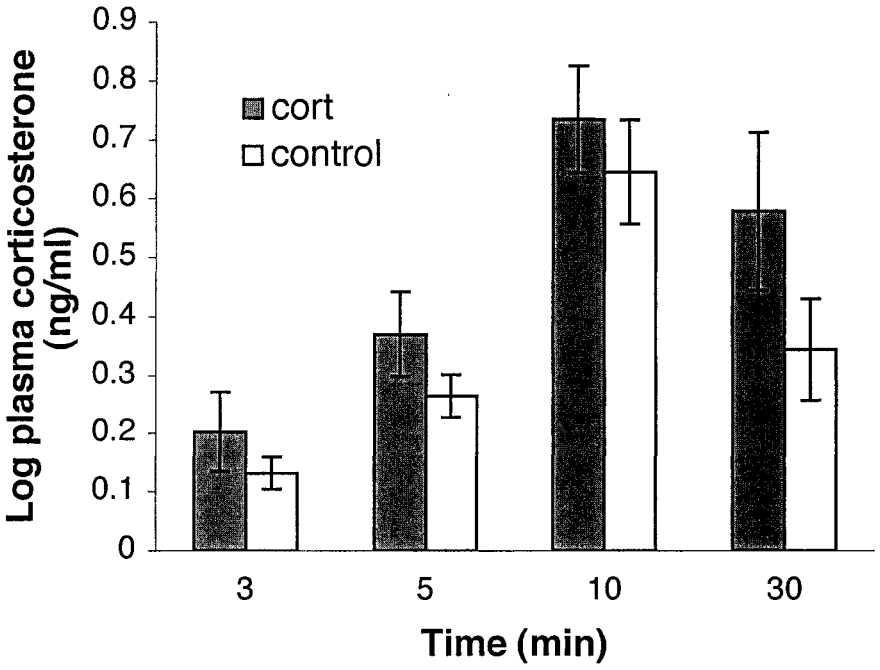


Figure 2.5: When subjected to a capture and restraint protocol adult quail from Cort-implanted mothers had higher levels of plasma corticosterone than controls ($t = 2.5$; $p = 0.02$). Sample sizes were nine corticosterone and ten control.

Chapter III: Sex Differences in the Organizational Effects of Yolk Corticosterone in Quail

INTRODUCTION

Among disparate vertebrate taxa, maternal stress has permanent effects on offspring physiology, morphology, and/ or behavior. For example, in rodents, maternal stress results in offspring that exhibit enhanced anxiety behavior, reduced learning ability (Weller, *et al.* 1988, Vallee, *et al.* 1999), heightened responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis (Peters 1982, Takahashi, *et al.* 1988, 1992a,b, Henry, *et al.* 1994, Fride, *et al.* 1986, Maccari, *et al.* 1995), and diminished reproductive capacity (Herrenkohl 1979, 1986), In fish, elevated maternal cortisol causes larvae to grow more slowly (McCormick 1999) and have more abnormalities (Morgan, *et al.* 1999) than controls. In common lizards (*Lacerta vivipara*), maternal stress results in reduced offspring dispersal (De Fraipont, *et al.* 2000). In domestic chickens (*Gallus gallus domesticus*) increased corticosterone in eggs decreases growth and increases fluctuating asymmetry (Eriksen, *et al.* 2003). Moreover, in Japanese quail (*Coturnix japonica*), elevated maternal corticosterone reduces offspring growth and heightens adult responsiveness of the HPA axis (Hayward & Wingfield 2004).

In some cases, these effects have been attributed to the embryo's exposure to elevated maternal glucocorticoids during development (Barbazanges, *et al.* 1996), although increases in the steroid alone,

without the experience of stress in the pregnant dam, will not recreate the effects observed as a result of maternal stress (Szuran, *et al.* 1994, Holson, *et al.* 1995). For example, maternal stress during pregnancy increases the responsiveness of the HPA axis in rats (reviewed above) but elevating corticosterone in pregnant rats with implanted pellets decreased offspring HPA responsiveness (Szuran, *et al.* 1994) and corticosterone injections in pregnant rats had no effect on offspring HPA responsiveness (Holson, *et al.* 1995, reviewed in Welberg & Seckl 2001).

Recently, it has been shown that high maternal corticosterone in Japanese quail is transferred to egg yolk, and that chicks from eggs laid by females with elevated corticosterone grow more slowly than controls and have higher levels of plasma corticosterone in response to capture and restraint as adults (Hayward & Wingfield, 2004). This study addresses whether yolk corticosterone itself is responsible for the observed effects of elevated maternal corticosterone on offspring. Therefore, we made two predictions:

1. Elevating yolk corticosterone concentration prior to incubation would slow chick growth.
2. Elevating yolk corticosterone concentration would heighten adult stress response.

The first prediction was based on the fact that elevated maternal corticosterone slowed chick growth (Hayward & Wingfield 2004), and also on the fact that slow growth is associated with heightened anxiety and heightened sensitivity of the HPA axis in Japanese quail (Jones, *et al.* 1992, Jones, *et al.* 1997). If females in territories with high predator density have high circulating glucocorticoids (Scheuerlein, *et al.* 2001, Silverin 1998, Clinchy, *et al.* 2004), it may be adaptive for offspring to

exhibit heightened wariness or anxiety when exposed to high levels of glucocorticoids in egg yolk.

From both proximate and ultimate perspectives, there are reasons to also predict that high maternal corticosterone in yolk will sensitize the hypothalamic-pituitary-adrenal (HPA) axis of developing birds. Furthermore, prenatal stress is known to sensitize the HPA axis of developing rodents (Henry, *et al.* 1994, Takahashi, *et al.* 1992a, b) by reducing the number of mineralocorticoid receptors in the hippocampus and thereby reducing the potential for negative feedback control of glucocorticoid secretion (Arriza, *et al.* 1988, Maccari, *et al.* 1994, Barbazanges, *et al.* 1996).

To test our two predictions about the effects of high yolk corticosterone we injected eggs with corticosterone prior to incubation and later quantified growth rates and adult stress response in the quail from these eggs.

METHODS

Egg injections:

To determine a biologically relevant quantity of corticosterone to inject into Japanese quail eggs we first determined the average endogenous corticosterone and its standard deviation in 12 fresh eggs shipped from Boyd's Birds, Pullman, Washington (the source of all quail used in our experiments). Average endogenous yolk corticosterone concentration in Boyd's strain of Japanese quail was determined to be 1.11 ± 0.30 ng/g yolk. The average egg yolk mass (calculated by separating the yolk from the other egg constituents and weighing it) was 2.99 g. Therefore, we calculated that we would need to inject 1.79 ng

corticosterone to increase the overall yolk concentration two standard deviations. After these injections final yolk concentrations would be 1.70 ng/g, comparable to the final concentration of 2.06 ng/g found in eggs laid by females with experimentally elevated corticosterone (see Chapter II).

Prior to injection, corticosterone was suspended in sterile peanut oil. Solutions of corticosterone in oil were made up by adding 8.94 μl of 10ng/ 1 μl stock to 500 μl oil that had been sterilized by autoclave. Control oil was prepared the same way without having corticosterone added to it. Injections were conducted by sterilizing a small area of egg shell with alcohol and using a 26 gauge butterfly infusion set with 10 ml syringe to slowly inject 10 μl of oil into the center of each egg. Punctures were sealed with transparent wound dressing as described in Chapter II.

Incubation and Hatching:

Once injected, eggs were placed in one of two Lyon TX7 Auto-turn incubators and maintained at 38°C and 50% humidity while being turned hourly. Treatments were interspersed between the two incubators at random. Three days before hatching, eggs were transferred to a brooder that maintained the same environmental conditions as the incubator but without turning the eggs. Within the brooder eggs were placed in a grid constructed of wire mesh and cardboard dividers so that the identity of the chicks could be established post-hatching.

Chick-rearing and comparison of adult physiology:

After hatching, chicks were marked with colored leg bands and placed into cohorts comprised of five treated and five control birds. Cohorts were reared in large pens with heat lamps, ad libitum food (De

Young's game bird starter crumble) and water. Mass measurements were taken every day within the same hour.

Once chicks reached sexual maturity at eight weeks of age, we measured activity of the hypothalamic-pituitary-adrenal axis by obtaining a stress series. Small blood samples of about 60 μl were taken from the alar vein before three, and at five, ten and 30 minutes from entering the chamber where the quail were housed. Between blood draws quail were restrained in soft cotton bags with minimal stimulation. Concentrations of corticosterone were calculated using radioimmunoassay described in Chapter II.

Analysis:

Chick growth rates were compared by calculating a slope of growth between days two and six by linear regression for each chick and comparing slopes between treatments with a t-test. The two to six day portion of the growth curve was used for the regression because it is a steep, almost linear phase of growth where differences in growth rate are most pronounced. Sample sizes for the growth analysis were 17 control males, 25 corticosterone-treated males, 24 control females, and 23 corticosterone-treated females.

To analyze treatment effects on baseline plasma corticosterone concentrations we used a two-way ANOVA with treatment and sex as factors. To compare response to capture and restraint, we calculated the area under each curve and compared between treatments and sexes with the same type two-way ANOVA. Using area under the curve provides an integrated measure of HPA activity during the entire period of restraint (Bruener, *et al.* 1999). Sample sizes for these analyses were ten

corticosterone-treated males, 13 control males, 15 corticosterone-treated females and 14 control females.

RESULTS

Effect of elevated yolk corticosterone on offspring growth:

Male chicks that hatched from eggs injected with corticosterone grew more slowly than male controls ($t = -2.876$; $p = 0.006$; Fig. 3.1). Among females, however, there was no significant effect of corticosterone injection on growth rate ($t = -1.180$; $p = 0.24$; Fig. 3.2).

Effect of elevated yolk corticosterone on adult stress response:

There was no treatment effect or sex difference in baseline corticosterone ($F = 0.20$; $p = 0.65$ and $F = 0.12$; $p = 0.73$ respectively). While there was also no overall effect of treatment on the magnitude of stress response ($F = 0.53$; $p = 0.47$), sexes differed significantly ($F = 4.10$; $p = 0.04$), and there was a significant interaction of treatment and sex ($F = 5.69$; $p = 0.02$). Corticosterone treatment decreased stress response in females (Fig. 3.3) but not in males (Fig. 3.4).

DISCUSSION

Interestingly, elevated yolk corticosterone slowed growth in males (in accordance with our prediction) but not in females. This finding is consistent with work done by Love (2003) in starlings, showing that corticosterone implants in laying females slowed growth in male but not female offspring. If we grouped the sexes to analyze growth, the strength of the effect on males would be enough to show significance for an

overall treatment effect, which likely explains why such effect was documented in Chapter II, although sexes were not analyzed separately.

Although sex-specific organizational effects of glucocorticoids are common (McCormick, *et al.* 1995, Lui, *et al.* 2001, Love & Williams 2003), little is known about the mechanisms responsible for these differences. Possibilities include sex differences in glucocorticoid receptor expression, sex differences in production of enzymes to break down corticosterone in the system (enzymes such as 11-βHSD) or sex differences in levels of circulating binding globulins that prevent corticosterone from entering cells or binding with receptors.

Our prediction that elevated yolk corticosterone would increase plasma corticosterone response to capture and restraint found no support. In fact, while the stress response was relatively low in all our quail (likely as a result of daily handling associated with growth measurements), elevated yolk corticosterone decreased the responsiveness of the HPA axis in females. This contrasts with the increased HPA responsiveness found in Japanese quail offspring of mothers with experimentally elevated corticosterone (Hayward & Wingfield 2004). The disparity between the effects of elevated maternal corticosterone (which is transferred to yolk) and experimentally elevated yolk corticosterone may be explained by differences in the distribution of corticosterone in the egg when injected as opposed to when deposited by the mother. Despite being lipid soluble, steroids are not distributed uniformly in yolk (Hackl, *et al.* 2003). Differences in distribution may cause differences in the timing of embryonic exposure to corticosterone. In rodents, it has been shown that timing of exposure to prenatal stress is a critical factor in determining the organizational effects exhibited by offspring (Welberg & Seckl 2001). An alternate explanation for the

difference in effect is that elevating plasma corticosterone in a laying female may alter other egg constituents, which, themselves alone, or in combination with elevated yolk corticosterone, act to increase offspring HPA responsiveness.

Additionally, the different organizational effects on the activity of female HPA responsiveness may relate to differences in the reproductive stage of the females at the time of sampling. Lui, *et al.* (2001) have shown that, in guinea pigs, there is a significant interaction of female reproductive stage and prenatal exposure to glucocorticoids on the activity of the HPA axis. Females exposed to synthetic glucocorticoids prenatally demonstrate heightened basal activity of the HPA axis during the early luteal phase and depressed HPA activity during the late luteal phase relative to controls (Lui, *et al.* 2001).

Interestingly, Lui, *et al.* (2001) also showed a sex difference in the organizational effect of prenatal glucocorticoid exposure on HPA activity. Male guinea pigs showed consistently reduced activity of the HPA axis. A separate study showed prenatal stress to increase HPA activity in female rats and decrease HPA activity in males (McCormick, *et al.* 1995). These studies emphasize the importance of considering sex and reproductive stage when evaluating the organizational effects of glucocorticoids.

Whether the effects of elevated yolk corticosterone are adaptive or not is difficult to address. Although many maternal effects have evolved as a way for mothers to program their offspring to maximize success in a dynamic environment (Mosseau & Fox 1998), other maternal effects are merely constraints of physiology (Dufty, *et al.* 2002). The maternal effects commonly associated with exposure to elevated glucocorticoids (including slow growth, increased sensitivity of the HPA axis and increased anxiety behavior) are generally considered deleterious.

However, more work needs to be done to evaluate the adaptive value of these traits in the context of a natural system, for example, by combining hormone manipulations with cross-fostering or relocation experiments to assess the adaptive value of altered phenotypes under different environmental conditions (Dufty, *et al.* 2002).

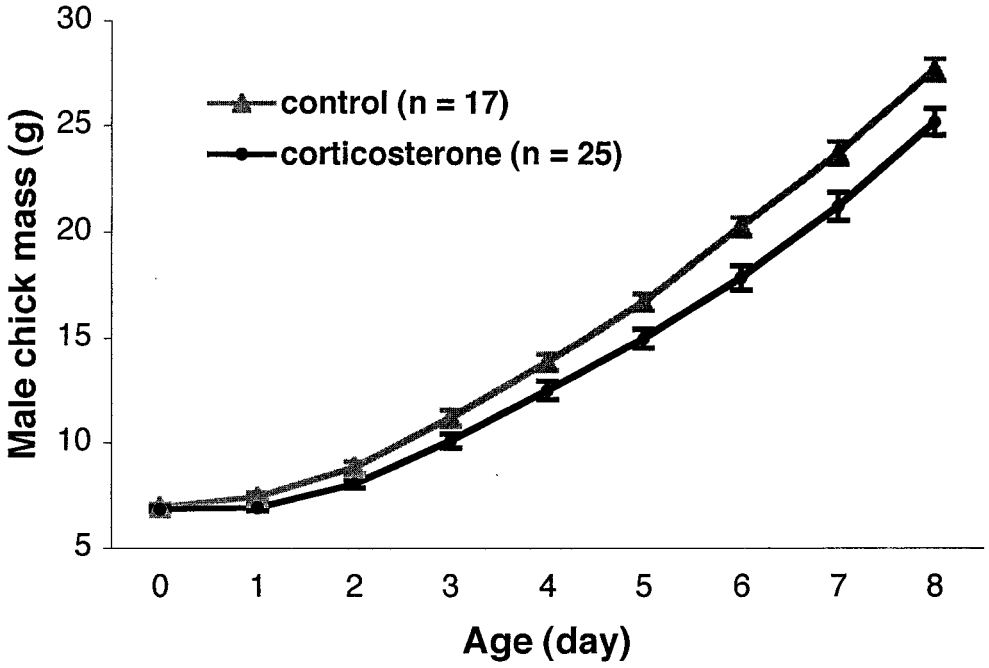


Figure 3.1:

Male chicks that hatched from eggs that had been injected with corticosterone grew faster in the first week than control chicks.

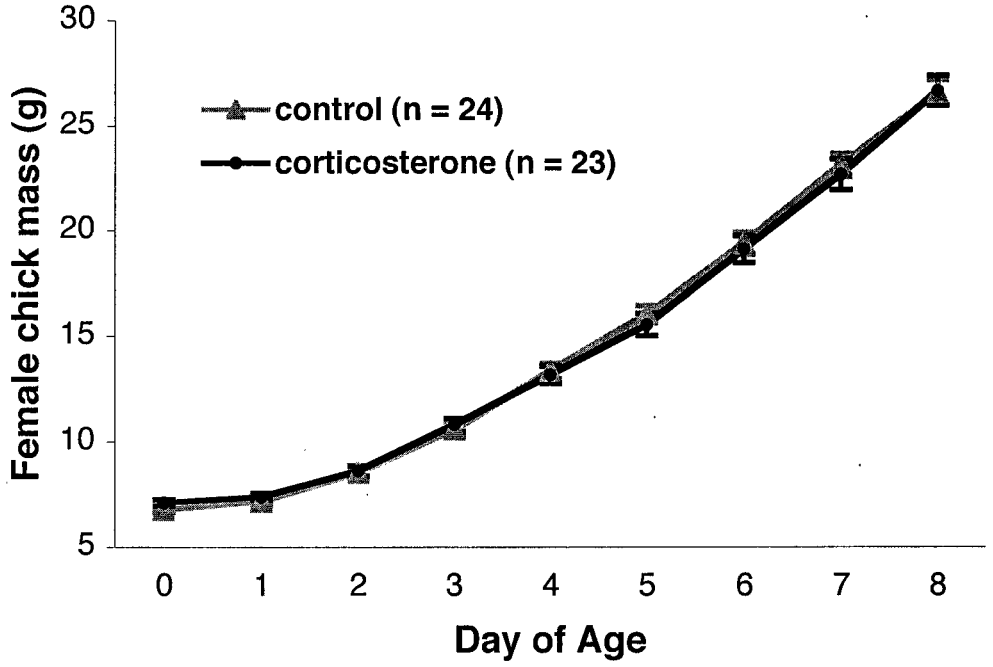


Figure 3.2:

There was no effect of corticosterone treatment on growth in female chicks.

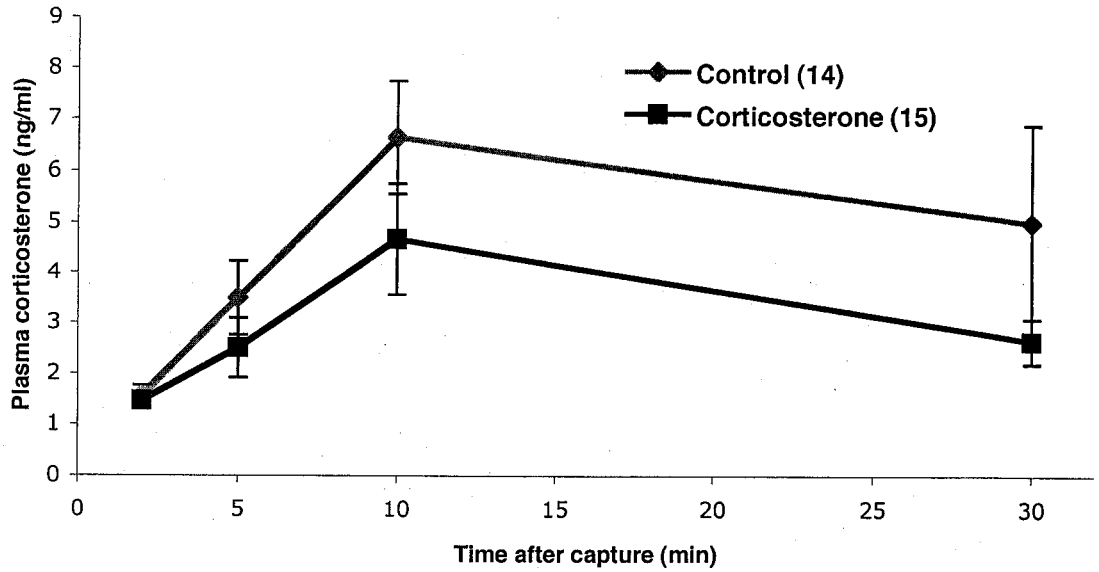


Figure 3.3:

Adult female quail from eggs injected with corticosterone released more plasma corticosterone in response to capture and restraint than controls.

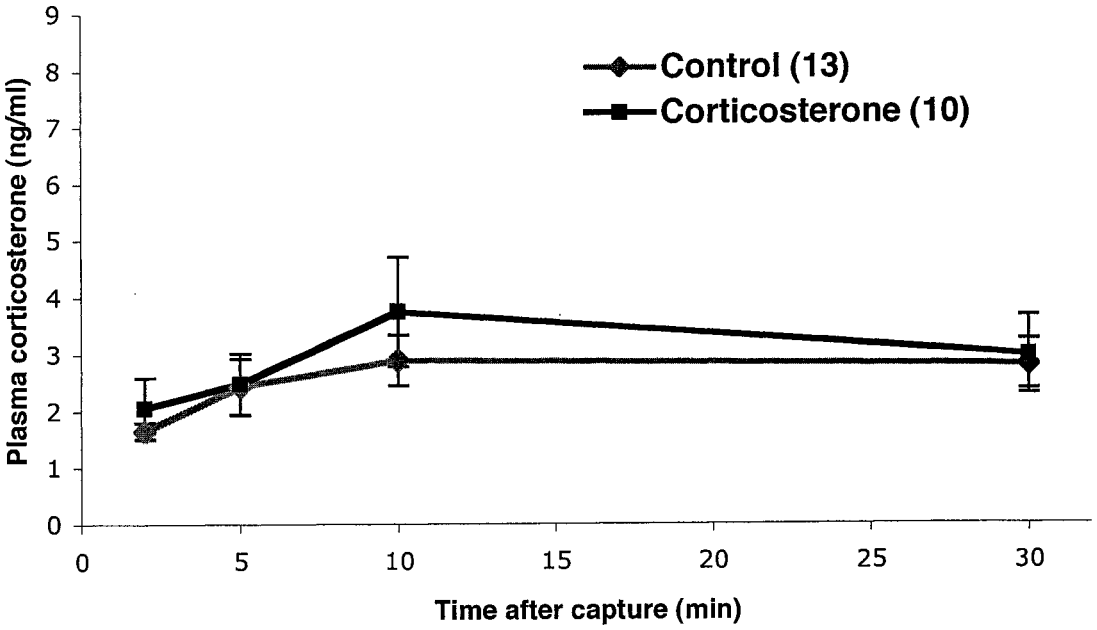


Figure 3.4:

There was no effect of corticosterone treatment on the plasma corticosterone response to capture and restraint in adult male quail.

Chapter IV: Effects of Elevated Yolk Corticosterone on Learning and Anxiety Behavior in Quail

INTRODUCTION

Environmental variation during embryonic development is known to permanently alter physiology and/or behavior in many vertebrate species. Maternal steroids and temperature are two examples of non-genomic factors involved in the *perinatal programming* by which physiological systems are organized during early development. Although perinatal programming may allow an organism to respond to cues about the local environment by altering life history strategy to maximize fitness under prevailing conditions (Clark & Galef 1995), the effects of maternal stress often appear deleterious (Welberg & Seckl 2001).

For example, in mammals, exposure to high levels of maternal glucocorticoids is associated with heightened anxiety (Fride, *et al.* 1986), increased responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis (Henry, *et al.* 1994, Takahashi, *et al.* 1992a,b), increased anxiety behavior (Fride, *et al.* 1986) abnormal sexual behavior (Ward 1972), reduced fertility (Herrenkohl 1979) and impaired learning ability (Vallee, *et al.* 1999, Weller, *et al.* 1998; for reviews see Herrenkohl 1986; Welberg & Seckl 2001). Less is known about the organizational effects of high maternal glucocorticoids in oviparous species, although maternal stress correlates with reduced larval hatch size in tropical damselfish, *Pomacentrus amboinensis* (McCormick 1998, 1999), reduced dispersal in common lizards, *Lacerta vivipara* (De Fraipont, *et al.* 2000), increased fluctuating asymmetry, increased mortality and slowed growth in domestic chickens, *Gallus gallus domesticus* (Eriksen, *et al.* 2003) and

reduced growth and heightened responsiveness of the HPA axis in Japanese quail, *Coturnix coturnix japonica* (Hayward & Wingfield 2004). Application of exogenous corticosterone to young chicks has been shown to impair subsequent learning in kittiwakes, *Rissa tridactyla* (Kitaysky, *et al.* 2003).

This study tested three predictions. The first was that elevated corticosterone in yolk would impair chick learning. The second prediction was that elevated yolk CORT would increase adult anxiety behavior. And the third prediction was that the effects of yolk corticosterone would vary with incubation regime. In order to assess the adaptive value of the phenotypic changes resultant from maternal stress, the effects of hormonal manipulations should be tested under a range of relevant environmental conditions (Dufty, *et al.* 2002). It is possible that, while the organizational effects of maternal stress appear detrimental in the lab (as most do, see above), they may provide an advantage under more challenging circumstances. For example, heightened anxiety may seem deleterious under lab conditions, particularly when it is associated with reduced growth and fertility, yet may provide a survival advantage in an environment with high predator density (Koolhaas, *et al.* 1999).

There are several reasons to hypothesize that elevated maternal corticosterone in yolk will increase anxiety behavior in avian offspring. First, prenatal stress increases anxiety in mammals (Fride, *et al.* 1986). Second, elevated maternal corticosterone increases the responsiveness of the hypothalamic-pituitary-adrenal axis and slows growth in Japanese quail (Hayward & Wingfield 2004). In Japanese quail both slowed growth and heightened sensitivity of the HPA axis are positively associated with anxiety behavior (Jones, *et al.* 1997). Possibly

heightened anxiety and HPA sensitivity result in slowed growth because they are energetically costly to maintain. And finally, from a theoretical perspective, high maternal corticosterone may signal poor habitat quality generally, and high predator density specifically (Silverin 1998, Scheuerlein, *et al.* 2001), so a more "reactive" coping strategy may help an offspring survive under suboptimal conditions (Koolhaas, *et al.* 1999). Of course, in order for a cue from the mother about an environmental condition like high predator density to phenotypically engineer offspring in an adaptive manner, there must be a high probability of the offspring confronting the same challenges themselves. In the case of a precocial chick, the predator density experienced by a laying mother will likely relate directly to the predator density in the environment where the chick will grow up.

The "tonic immobility" response refers to an anti-predator strategy in which quail temporarily suspend their righting response when confronted with a threat, essentially "freezing" or "playing dead". Both the ease with which tonic immobility can be induced in a quail and the duration of time they remain immobile are positively associated with anxiety or underlying fearfulness (Gallup 1979, Jones 1986, 1987, Jones, *et al.* 1997). Among vertebrates more generally, proclivity to freeze is considered to be a feature of the "reactive" coping strategy theorized to be adaptive under suboptimal conditions (Koolhaas, *et al.* 1999).

By testing response to a novel object we aim to assess exploratory and avoidance tendencies. Such tests have been used previously to test for fear in Japanese quail and domestic hens (Jones 1987, Jones, *et al.* 1996, 1997). Further, across vertebrate taxa, reduced exploratory and heightened avoidance behaviors are classic features of the reactive coping strategy (Koolhaas, *et al.* 1999).

We predicted that increasing yolk corticosterone by injecting it into eggs prior to incubation would have the following three effects on offspring anxiety behavior:

1. Increasing yolk corticosterone would significantly increase the anxiety demonstrated by adult offspring upon exposure to a novel object.
2. Increasing yolk corticosterone would facilitate elicitation of a "tonic immobility" response in adult offspring.
3. Increasing yolk corticosterone would prolong the "tonic immobility" response of adult offspring.

Furthermore, in many oviparous species, incubation temperature can have profound organizational effects. For example, effects of ambient temperature on an embryo's enzymes, hormones and receptors dictate which gonad-determining genes will be activated, and thus the sex of the individual (Norris 1996). Prior to hatch, avian embryos respond to fluctuations in incubation temperature with transitory peaks in corticosterone titers (Jacobs 1996), although it is unknown whether exposure to such increases in circulating corticosterone has long-term effects of offspring phenotype, nor whether there are synergistic effects between maternal corticosterone in yolk and elevations in embryo corticosterone in response to stress.

Although manipulating predator density and testing survival rates was beyond the scope of our study, it is not unlikely that incubation is more often disrupted or is interrupted for longer periods under poor environmental conditions (Hart, *et al.* 2002). Therefore, imposing a two and a half hour period of cooling prior to hatch is a biologically relevant environmental manipulation to use in conjunction with hormonal manipulations to test our third prediction.

METHODS

Yolk steroid manipulation:

All Japanese quail eggs were purchased from Boyd's Birds of Pullman, Washington and shipped second day mail to the University of Washington on the day of laying. In order to calculate doses for corticosterone injections, yolk mass and endogenous yolk corticosterone were measured from 13 eggs. To obtain yolk mass we separated the yolk from the albumin and weighed it. Yolk was then homogenized with an equal part water and run in 50 μl samples through extractions with diethyl ether, ethanol, and columns of diatomaceous earth before corticosterone levels were measured with radioimmunoassay as described in Hayward and Wingfield 2004 (using a protocol developed by Wingfield & Farner 1975 and modified by Schwabl 1993).

Average yolk mass was calculated to be 2.99 g and average yolk corticosterone concentration was 1.10 ng/g yolk with a standard deviation of 0.30. From these values we calculated the amount of corticosterone needed to increase average yolk corticosterone concentration by one and two standard deviations (0.89 ng and 1.79 ng respectively). These increases would bring yolk corticosterone concentrations to 1.40 and 1.70 ng/g, levels well within the range of those measured in eggs laid by female Japanese quail given corticosterone implants that brought their plasma corticosterone to titers achieved in response to capture and restraint (Hayward & Wingfield 2004)

For the experiments, Japanese quail eggs were shipped in batches of 60 eggs on the day of laying. Upon arrival, eggs were stored at room

temperature under damp paper towels until injections could be completed and incubation initiated. Eggs were injected with 10 μl sterile peanut oil containing either 0.89 ng corticosterone (1 st dev), 1.79 ng corticosterone (2 st dev) or no steroid (control). Injections were performed with 26 gauge butterfly needles (Abbott Laboratories, Chicago, Illinois, USA) and a 5 ml syringe, held so as to inject the center of each yolk. After injection, holes were sealed with small patches of OpSite transparent wound dressing (Smith&Nephew Medical Limited, Hull, England) and eggs labeled.

Incubation and temperature manipulation prior to hatch:

Eggs were incubated in an auto-turning Brinsea Octagon 250 incubator at 37° C with about 50% humidity for 15 days before being moved to a brooder where they were incubated at the same temperature and similar humidity without being turned until hatch. The brooder was divided into compartments to ensure proper identification of chick treatment post-hatch. In the second experiment eggs were injected with corticosterone or control oil (as described above) and cooled to room temperature (22°C) for two and a half hours prior to hatch on day 16 of incubation (after the first chicks had begun to pip) before being returned to the brooder. While being cooled, eggs were stored in open-top cardboard egg carton covered with a damp paper towel.

Chick rearing:

Upon hatch, chicks were weighed and assigned a unique combination of colored leg bands. Chicks were reared under heat lamps in groups 10-15 same-age, mixed treatment individuals with water and

De Young game bird starter crumble provided *ad libitum.* All chicks were weighed at ten o'clock every morning for their first ten days.

Learning

Learning test arena: The test arena was located in a soundproof environmental chamber in a different room of the building where chicks were housed. The arena was designed based on specifications published by Regolin and Rose (1999) and consisted of a cardboard box, 60 cm long x 50 cm wide x 50 cm high, in the center of which was placed a smaller box (20 cm x 20 cm x 20 cm) open at the back to form a U-shaped barrier. At the front of the barrier was a mesh window. Attached to the outside of the large box, opposite the window in the barrier, was a second small box (30 cm x 20 cm x 20 cm) with a second mesh window where the three same-age conspecific escorts were placed so as to be visible to the test subject during the trial. Thus, the boxes comprised a simple maze wherein the test subject was forced to navigate around one of the U-shaped barrier's sides to be reunited with its cohort (Fig. 4.1).

The arena was lit with two heat lamps, one centered over the U-shaped barrier and one above the cohort's outer box. Observers and video camera were situated behind and above the light sources so as to be inconspicuous to test subject. The floor of the arena was covered with the same white wood shavings as the chicks' home cage.

The behavioral trials: At two days of age, chicks were transported in an opaque box with three same-age conspecifics to the learning test arena. A trial was conducted by removing the test subject from the box with its cohort and placing it in the center of the U-shaped barrier, facing the mesh window through which the cohort in the outer box was visible.

After navigating the maze a first time, chicks were left in proximity to the cohort for ten seconds. If a chick failed to navigate the maze within ten minutes, they were removed from the study and recorded as a failure. For chicks that completed the maze, second trials were conducted 30 minutes from the time of the first trial. During each trial, we recorded the time taken for the chick to cross the left or right side of the mesh window in the outer box. We also recorded the latency to first contact call made by the test subject and direction taken to navigate the maze (left or right). Trials were taped, and tapes later analyzed to quantify contact calling. Trials were conducted when chicks were two or three days of age. The cohort was varied, but the same chicks were often used as a cohort for several consecutive trials. Chicks used in cohorts were either not tested at all for learning or had already completed both trials one and two.

Analysis of learning: We found high variability in the amount of time subjects took to recover from the shock of handling, and perceive the cohort from which they had been separated. In order to eliminate this variability, we used time from first contact call to maze completion rather than time from release for each bird. There was no treatment effect on latency to contact call (see results). There were no differences in maze performance among the control chicks of the different experiments, so they were lumped together for purposes of analysis. We log transformed finish times to homogenize variance among treatments. Each bird was assigned an improvement score by subtracting the time taken to complete the maze during the second trial from time taken to complete the maze during the first trial. ANOVA was used to compare latency to contact call in the first round, improvement scores, and time

to complete maze in trial one among treatments. Chi-square tests were used to test for treatment effect on consistency in turning left or right and on failure rates in trial one.

Anxiety Behavior

Novel Object test: At 25 days of age, and after one week of adjustment to individual housing, quails' cages were covered with newspaper so as to restrict visual contact with quail in neighboring cages, while leaving the cage front unobstructed. Twenty-four hours later (at 26 days of age) quail were presented with a 7mm x 4mm fluorescent orange fishing lure with eyes painted on it, which was hung from the front door of the cage. For three minutes after initial presentation of the novel object an observer recorded a behavioral score at ten second intervals using the following system (based on that developed by Jones, *et al.* 1997):

4 = "escape behavior" characterized as jumping, pacing at the back of the cage, or hiding behind the food dish at the back of the cage.

3 = facing the back of the cage while not engaged in escape behavior or pacing along the sides of the cage.

2 = eating, drinking, bathing, reclining, or facing the sides of the cage.

1 = looking at the novel object.

0 = pecking or looking at the novel object while close enough to peck.

Seventy males were tested for response to novel object. These included 27 from corticosterone-injected eggs uncooled prior to hatch (uncooled CORT), 25 uncooled control, eight from corticosterone-injected eggs cooled for two and a half hours prior to hatch (cooled CORT) and 10 cooled control. Eighty females were tested (31 uncooled CORT, 35

uncooled control, five cooled CORT, and nine cooled control). A subset of these quail comprised of 45 males (16 uncooled CORT, 11 uncooled control, eight cooled CORT, and 10 cooled control) and 56 females (20 uncooled CORT, 22 uncooled control, five cooled CORT and nine cooled control) were assessed for "baseline" anxiety behavior by three minutes of observation prior to presentation of novel object. During this pre-observation period, anxiety behavior was scored as above, but with a score of one assigned when quail were looking at the door of the cage or at the investigator and a score of zero assigned when quail were close enough to the door at the front of the cage to peck the novel object if it were hanging there. For both the pre-observation period and the novel object test, the observer recorded whether or not the quail either jumped or pecked at the novel object. Jumping was assumed to be the strongest escape response exhibited and pecking the most highly exploratory.

Anxiety behavior analysis: For analysis, the 18 scores for each three-minute observation period were summed (one score between zero and four for each ten second interval), and then compared using a Kruskal Wallis test, after finding that data were not normally distributed. Mann-Whitney U tests were used to test for sex differences in anxiety behavior. Sexes did not differ in baseline anxiety ($U' = 1398.5$; $p = 0.34$) nor in response to novel object ($U' = 2958.5$; $p = 0.55$) so were combined for analysis of treatment effect. A Chi-square test was used to determine whether there was a difference between treatments in the occurrence of jumping and pecking.

Tonic Immobility Test: At 29 days of age, quail were removed from their cage and transported to an adjacent room for assessment of their tonic

immobility response (following protocol outlined by Jones, *et al.* 1997). Briefly, quail were placed on their backs in a V-shaped Styrofoam "bed", and gently restrained with a hand over their sternum for 15 seconds, at which point, quail were slowly released and left to lie immobile on their backs. If the quail righted itself within 15 seconds of release, the procedure was repeated. If, after five attempts, the quail would not lie still for longer than 15 seconds after release, it was considered non-susceptible. Once the quail lay still for 15 seconds, latency to righting was recorded for up to ten minutes. Five quail were excluded due to irregularities in handling prior to the trial; therefore, final sample sizes for the tonic immobility tests were 69 males (27 uncooled CORT, 25 uncooled controls, seven cooled CORT, and 10 cooled controls) and 76 females (28 uncooled CORT, 34 uncooled controls, five cooled CORT, and nine cooled controls).

Because of the non-normal distribution of the data, non-parametric statistics were used to analyze the results of tonic immobility tests. There was no sex difference in the number of inductions ($U' = 1853$; $p = 0.30$), so sexes were combined in a single Kruskal-Wallis test. Females tended to hold immobile for longer than males ($U' = 3072.5$; $p = 0.07$). Therefore, latency to righting was analyzed separately for males and females. After corticosterone treatment was found not to affect latency to righting, treated and control birds were grouped together to test for the effect of cooling using a Mann-Whitney U test.

RESULTS

Learning

Experiment 1: Treatment did not affect the percentage of chicks that failed to complete the maze on first trial ($\chi^2 = 0.39$; $p = 0.82$; Fig. 4.2). Latency to first contact call was not affected by treatment ($F = 0.84$; $p = 0.43$). Percentage of chicks taking a different direction to complete the maze their second trial (left rather than right or vice versa) was also unaffected by treatment ($\chi^2 = 1.14$; $p = 0.57$).

Treatment did have significant effects on time taken to complete the maze during first trial ($F = 9.90$; $p = 0.0002$; Fig. 4.3). Chicks that hatched from eggs injected with 0.89 ng corticosterone (enough to increase average endogenous levels by one standard deviation) completed the maze significantly faster than control chicks ($p < 0.0001$) and chicks from eggs injected with 1.79 ng corticosterone ($p = 0.001$).

Treatment also significantly affected the improvement rates of chicks from first to second trial ($F = 3.57$; $p = 0.01$; Fig. 4.3). Chicks from eggs injected with 0.89 ng corticosterone actually took longer to complete the maze on the second trial, showing significantly less improvement than control chicks ($p = 0.003$) and marginally less improvement than chicks from eggs injected with 1.79 ng corticosterone ($p = 0.10$).

Experiment 2: Treatment did not affect the percentage of chicks that failed to complete the maze on first trial ($\chi^2 = 1.32$; $p = 0.52$; Fig. 4.2). Latency to first contact call was not affected by treatment ($F = 1.45$; $p = 0.25$). Percentage of chicks taking a different direction to complete the

maze their second trial (left rather than right or vice versa) was also unaffected by treatment ($\chi^2 = 1.41$; $p = 0.49$; Table 2).

Neither cooling alone nor cooling in combination with elevated yolk corticosterone influenced time to completion during the first trial ($F = 1.37$; $p = 0.26$; Fig. 4.4). However treatment significantly affected the improvement rates of chicks from first to second trial ($F = 5.42$; $p = 0.007$; Fig. 4.4). Chicks from cooled control eggs did not improve in maze performance while control chicks did ($p = 0.002$). Chicks from eggs injected with corticosterone earned improvement scores that fell between those of cooled control chicks and control chicks without being significantly different from either group ($p = 0.14$ and $p = 0.13$ respectively).

Anxiety Behavior

Novel Object test: There was no effect of treatment on baseline anxiety behavior ($H = 1.70$; $p = 0.64$). Similarly, there was no treatment effect on response to novel object ($H = 1.47$; $p = 0.69$; Fig. 4.5). The percentage of quail that jumped did not differ between treatments ($\chi^2 = 1.31$; $p = 0.73$). Nor did the percentage of quail that pecked the novel object (for males $\chi^2 = 4.19$; $p = 0.24$).

Tonic Immobility test: There was no difference among treatments in the number of inductions required to elicit a tonic immobility response of longer than 15 seconds ($H = 1.31$; $p = 0.73$; Fig. 4.6). Similarly, there was no treatment effect on the latency to righting for either sex (for males $H = 632.5$; $p = 0.60$; for females $H = 2.41$; $p = 0.49$ Fig. 4.7). However, there was a trend for cooled quail to remain immobile for less time than uncooled quail ($U' = 2161.5$; $p = 0.07$)

DISCUSSION

Learning

Our results show that, while unmanipulated two-day old Japanese quail are capable of learning to navigate a simple maze, chicks subjected to elevated yolk corticosterone and/ or to a two and a half hour period of cooling prior to hatch show no such evidence of learning in equivalent trials. These results support our first prediction that elevated corticosterone prior to hatch would impair chick learning and are consistent with organizational effects of glucocorticoids documented in other species (see introduction). However, interpretation of these results is complicated by the fact that chicks from eggs injected with a low dose of corticosterone were significantly faster at completing the maze in their first trial. The fact that they failed to improve in the second trial does not put them at any real disadvantage relative to controls and may not represent reduced learning ability as much as a limit to the amount that they might be expected to reduce their time to completion.

Experiment one: Interestingly, chicks from eggs injected with a low dose of corticosterone prior to incubation completed the maze considerably faster than controls during their first trial, although their advantage was not maintained in the subsequent trial (Fig. 4.3). Because there was no difference in latency to contact call, it does not seem that the chicks differed in the level of anxiety they experienced upon separation from the cohort or in their degree of motivation to be reunited. We tentatively conclude that, while high levels of maternal corticosterone may incur costs associated with reduced learning ability in offspring, there may be advantages to small elevations of maternal corticosterone, including an

ability to rapidly navigate obstacles in a novel environment to prevent prolonged separation from the cohort. It should be re-emphasized that the apparently reduced learning ability may actually be due to the fact that the chicks were fast to begin with (as fast as the control chicks in their second trail) and may not represent a disadvantage.

Experiment two: Surprisingly, two and a half hours of cooling to room temperature prior to hatch significantly impaired chick learning (Fig. 4.4), most likely due to the organizational effects of elevated endogenous corticosterone. Chicks from cooled control eggs were significantly slower in the maze during their second trial than control chicks. This may be due to the organizational effects of elevations in corticosterone experienced prior to hatch in response to cooling (Jacobs 1996). Chicks from eggs injected with corticosterone prior to hatch, while not significantly faster in their second trial than their first, also did not significantly differ from controls in either their improvement score or their time to complete trial two. In contrast, cooled control chicks differed from control chicks in both of those parameters. Therefore, the results from experiment two suggest that elevated maternal corticosterone in egg yolk may protect a developing embryo from detrimental central effects of interrupted incubation. This possible protective role of corticosterone may be adaptive in an environment where maternal corticosterone is elevated due to reduced food abundance or high predator density, either of which condition might force a female to spend extended periods away from the nest during incubation.

Anxiety Behavior

We found no significant effect of elevated yolk corticosterone or interrupted incubation on anxiety behavior in quail. We did, however, find a trend ($p = 0.07$) for chicks that had been cooled prior to hatch to have a lower latency to righting in the tonic immobility test (Fig. 4.7). Although our power was low (0.05 for the novel object test, 0.36 for the tonic immobility test) our sample size would have allowed us to detect a treatment effect as small as 4.47 on anxiety score and 31.3 sec of time immobile. We used the protocols described by Jones et al. (1997), which proved to be appropriate for detecting significant line differences in the same species. We are confident that the egg injections succeeding in having physiological effects on growth (reported in Chapter III) and behavioral effects on chick learning so we can conclude that the lack of treatment effect is not related to a problem with injection technique.

In summary, we present evidence that, while elevated maternal corticosterone seems to impair learning in an avian system, it may also confer advantages in heightened navigational ability in a novel environment or in protecting from the detrimental effects of interrupted incubation. These and similar trade-offs must be investigated further to fully understand the organizational effects of elevated corticosterone in an evolutionary context. Despite precedent for maternal corticosterone to increase anxiety behavior in other vertebrates, we found it to have no effect on anxiety behavior in Japanese quail.

Table 2: There was no difference among treatment groups in the rate of failure during the first trial in the maze, the latency to contact call during the first trial, or in the percentage of chicks that took the opposite direction in the second trial from the direction they took in the first trial.

TREATMENT	Percent failed	Latency to first contact call (sec)	Percent to switch directions in 2nd trial
Control	28	1.29	34
1 St Dev CORT	21	1.19	44
2 St Dev CORT	23	1.24	27
Cooled control	19	1.19	18
Cooled 2 St Dev CORT	14	1.34	23
P-value	**0.84**	**0.09**	**0.59**

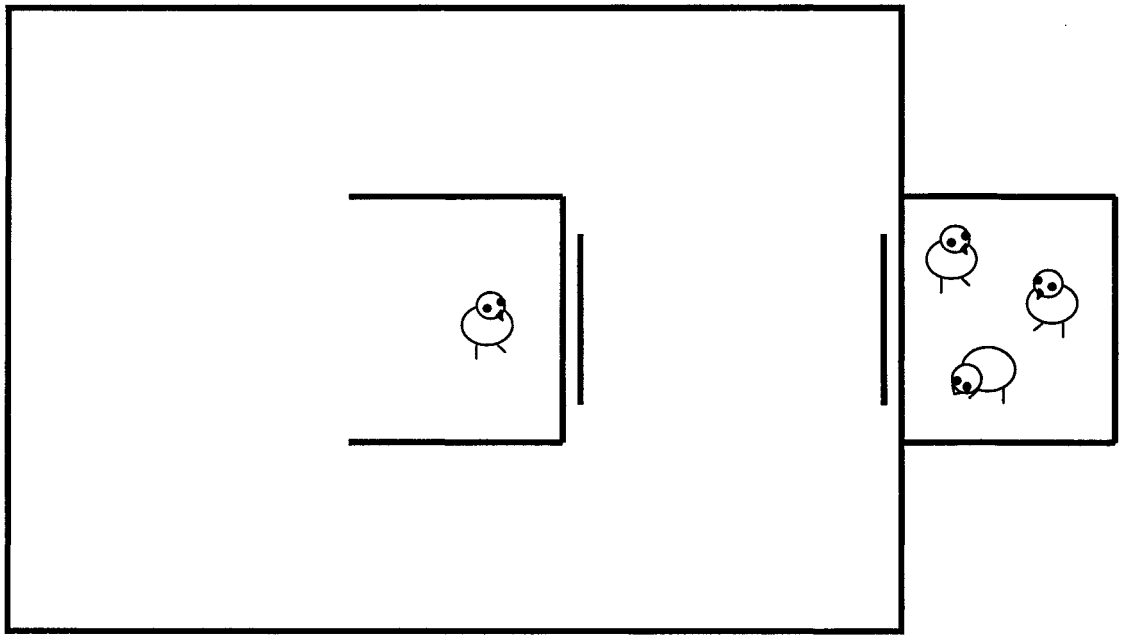


Figure 4.1: This schematic represents the maze arena with which we tested learning in two-day old quail chicks. The double lines represent mesh windows. The test subject was placed in the center of the U-shaped barrier at the beginning of each trial. Three same-age conspecifics in an outer compartment were visible to the test subject through the windows.

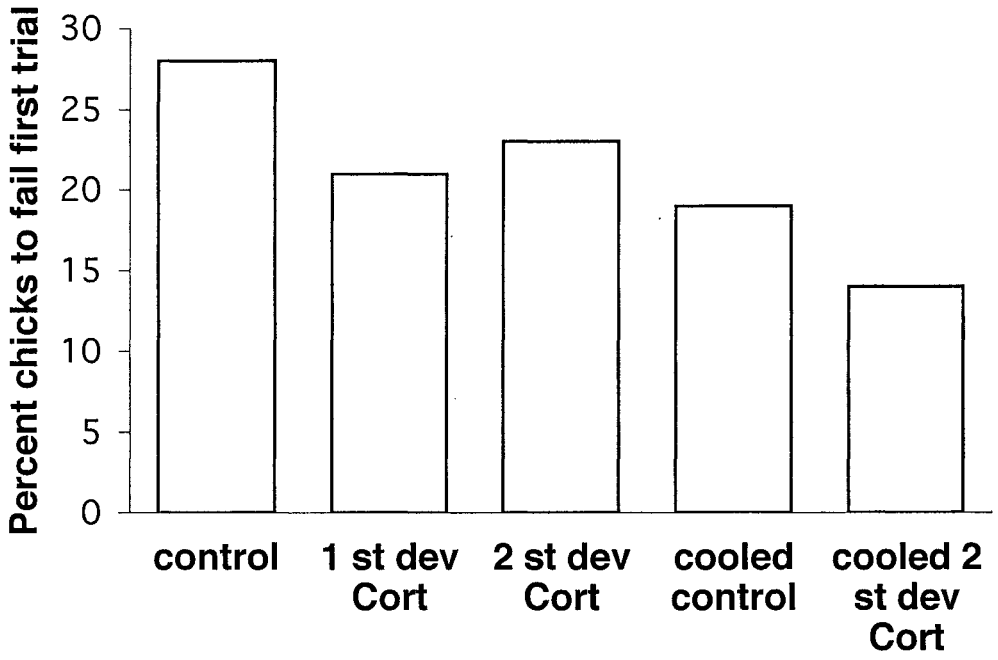


Figure 4.2: Although there was no significant difference in the percentage of chicks to fail their first trail among treatment groups, higher percentages of chicks exposed to elevated yolk corticosterone and/ or to cooling prior to hatch successfully completed the maze upon first trial. Sample sizes were 49 control, 22 1 st dev, 23 2 st dev, 14 cooled control, and 15 cooled 1 st dev.

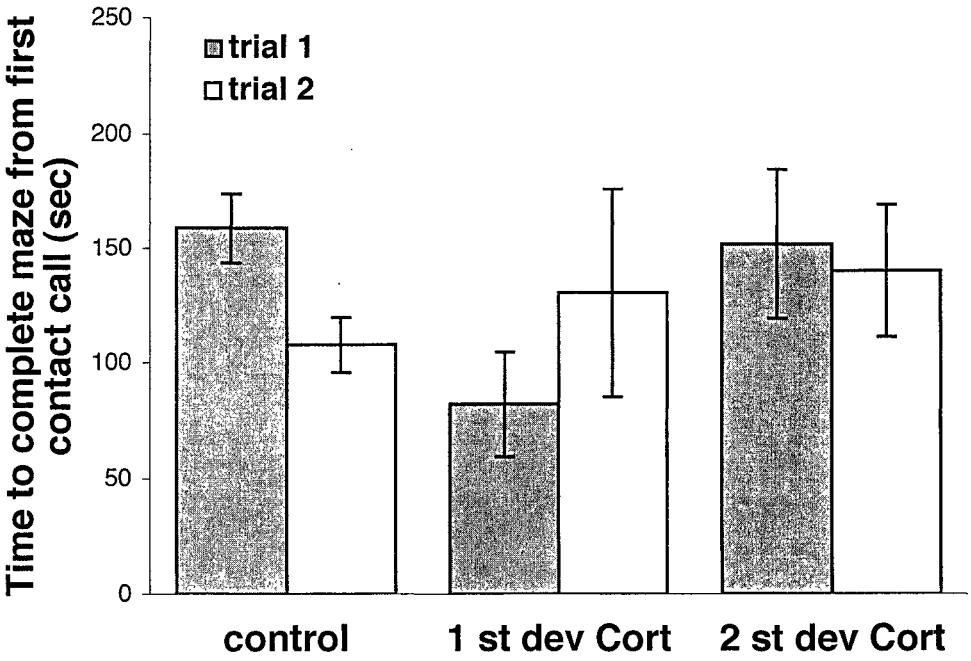


Figure 4.3: While chicks from eggs injected with 0.89 ng corticosterone (1 st dev) were significantly faster than controls and chicks from eggs injected with 1.79 ng corticosterone at completing the maze during trial 1, only control chicks became faster in their second trial than they had been in the first. Sample sizes were 35 control, 17 1 st dev, and 18 2 st dev.

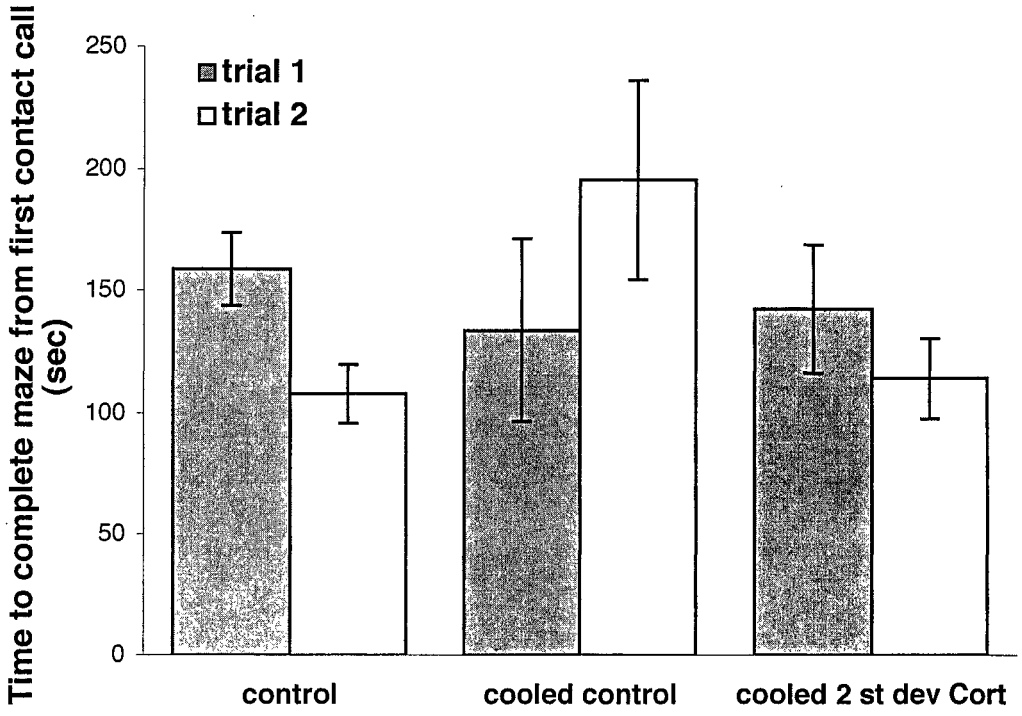


Figure 4.4: Chicks from eggs injected with 1.79 ng corticosterone and/ or cooled prior to hatch showed significantly less improvement in their second trial than control birds. Sample sizes were 35 control, 11 cooled control and 13 cooled, corticosterone injected.

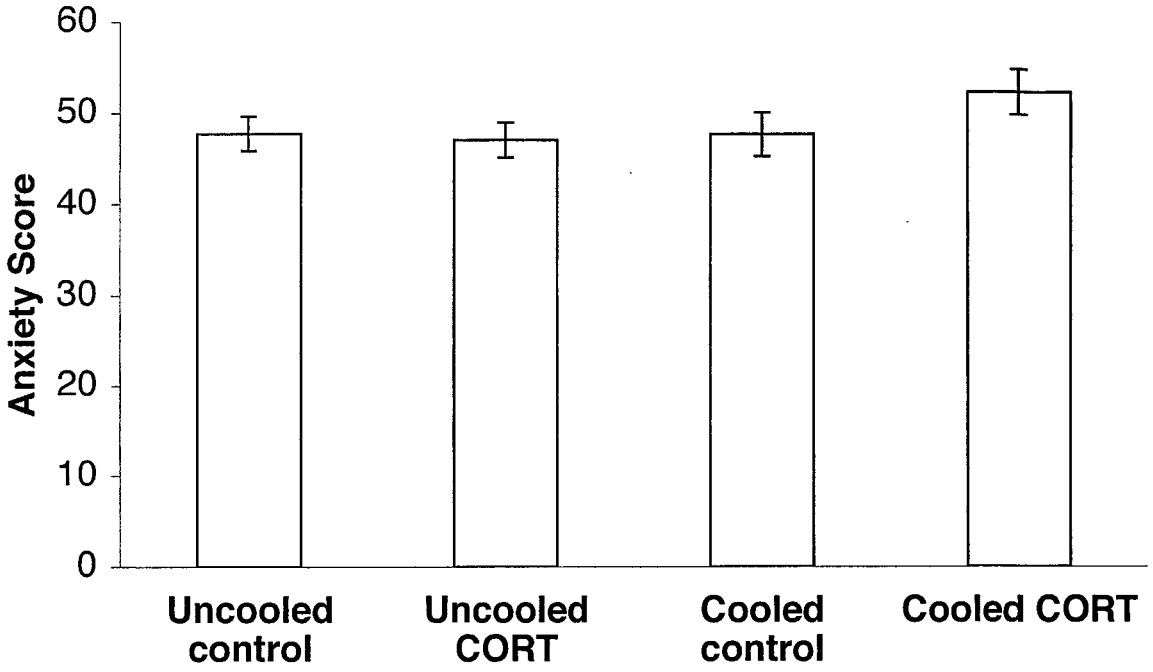


Figure 4.5: There was no treatment effect of anxiety score in response to exposure to a novel object.

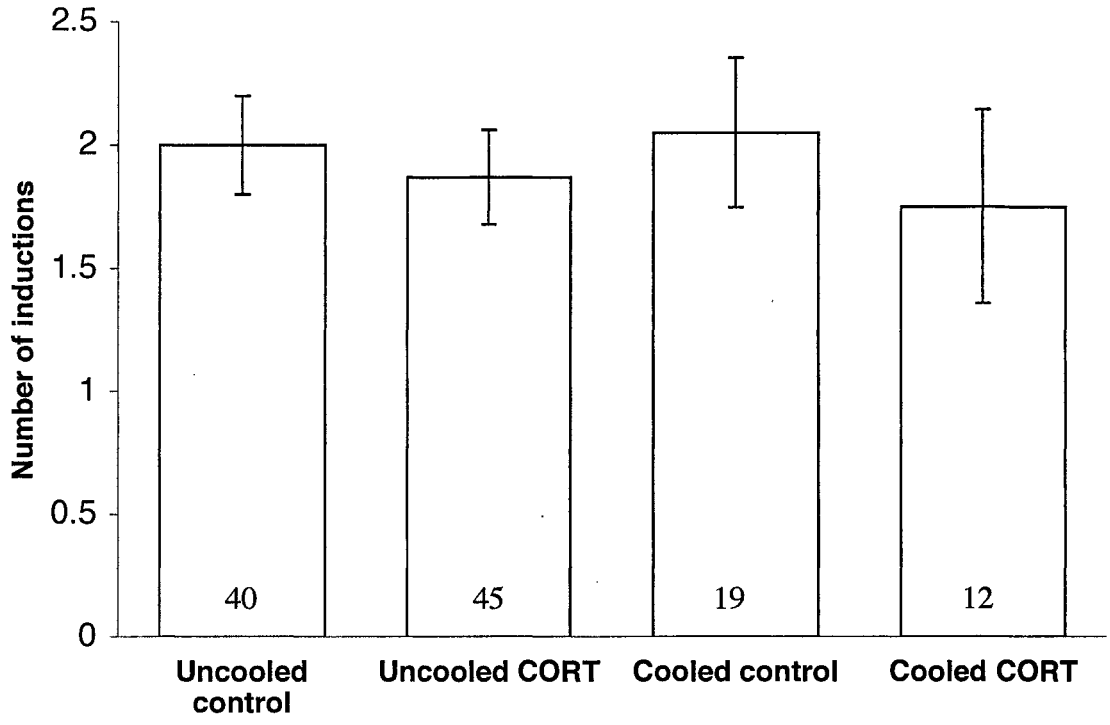


Figure 4.6: There was no treatment effect on the number of inductions required to elicit a tonic immobility response.

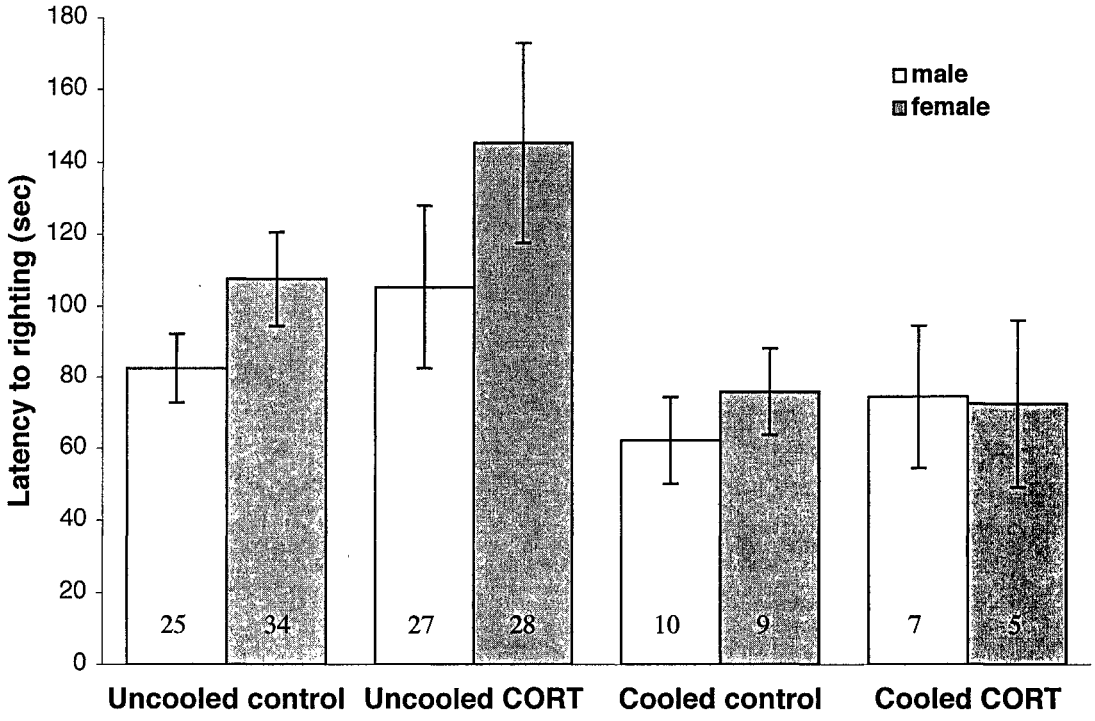


Figure 4.7: There was no effect of treatment on the latency to righting after tonic immobility had been induced.

END NOTES

While this research has shown for the first time that elevated plasma corticosterone is transferred to avian egg yolk, and that it has permanent organizational effects on offspring, it is still unclear whether these effects are adaptive, or merely the product of physiological constraint. The last question proved beyond the scope of this research. Future studies aimed at making this distinction will have to account for many complicating factors, some of which are discussed here.

First, the natural history of the species should be considered. In order for a cue about the environment of a laying bird to be relevant for her developing offspring, there must be a high likelihood of the offspring confronting the same challenges as their mother once they are out of the nest. If a bad storm raises corticosterone in a laying bird, there is little reason to expect that it will be adaptive for her offspring to alter their life history strategy as a result. On the other hand, if predator density is high on the territory of a non-migratory female with precocial young, then her high corticosterone at the time of laying may be a relevant cue for her offspring, and may be expected to program a strategy that increases likelihood of survival.

Second, yolk corticosterone manipulations, while an important early step in characterizing organizational effects, may not accurately simulate signals from mother to offspring during times when environmental conditions are challenging. This possibility is emphasized by the fact that elevated plasma corticosterone in female quail resulted in offspring with heightened hypothalamic-pituitary-adrenal response to capture and restraint, while elevated yolk corticosterone resulted in female offspring with lowered HPA activity. Similarly, different results

on mammalian offspring are achieved by exposing pregnant females to restraint stress than by elevating circulating glucocorticoids, although glucocorticoids are known to mediate many of those effects. Ideally, future studies will investigate the programming effects of maternal stress in birds by manipulating the environment of the laying female and testing for effects on egg constituents, incubation, parental behavior, and offspring phenotype. If the relative amounts of other yolk constituents change, or if parental behavior changes in response to challenging environmental conditions, then the effects of elevated yolk corticosterone on offspring should be evaluated in the context of these changes.

When assessing the adaptive value of the effects of maternal stress on offspring phenotype, it is important to consider trade-offs. While the offspring of control animals might always out-compete the offspring of experimentally stressed animals in a laboratory environment, the offspring of stressed individuals might out-compete controls in a natural environment with poor conditions. Along the same lines, traits should be evaluated as an integrated whole, rather than individually. Slow growth may be disadvantageous in and of itself, but it may be the cost of increased wariness or a superior ability to stay with the group, either of which may help a chick survive when predator density is high.

Finally, what is adaptive may differ depending on whether one takes the perspective of the mother or the perspective of the offspring. For example, in a polygynous species like Japanese quail, a mother in poor condition will do better to invest in daughters than in sons. The reason for this is that, in a polygynous species, competition for access to mates is stronger in males than in females. A male in poor body condition may not gain access to females at all, while all females will

likely breed. In this situation, it may benefit a mother to program slower growth in her male offspring than her female offspring, thus reducing competition for her daughters while they grow. Slow growth may, therefore, be seen as adaptive from the perspective of the mother, but maladaptive from the perspective of the male offspring. Any maternal effect should be evaluated similarly from the perspective of the two different generations.

In conclusion, it is my hope that the work presented here lays the groundwork for many future studies investigating the role of maternal corticosterone deposition in avian yolk in an ecological context.

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VITA

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