THE EFFECTS OF BREEDING DENSITY, YEAR, AND LABORATORY VS. FIELD ENVIRONMENTS ON PLASMA AND YOLK STEROIDS IN GREEN ANOLE LIZARDS (Anolis carolinensis)

By

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THE EFFECTS OF BREEDING DENSITY, YEAR, AND LABORATORY VS. FIELD ENVIRONMENTS ON PLASMA AND YOLK STEROIDS IN GREEN ANOLE LIZARDS (*Anolis carolinensis*)

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CHAPTER I

OVERVIEW

BACKGROUND

Maternal effects occur when the phenotype of an individual is determined not only by its own genotype and the environmental conditions it experiences during development, but also by the phenotype or environment of its mother (Mousseau and Fox 1998). A strong interest in maternal effects research developed during the middle of the twentieth century when studies began to demonstrate how human mothers’ diets and habits (e.g., smoking, alcohol consumption) affected the size and survival rate of their offspring (e.g., research into ‘fetal origins of adult disease’, or FOAD; Sapolsky 2004). Aside from the potential clinical value for understanding maternal effects, this research allows us more generally to better understand the full range of influences on offspring development and phenotypic expression.

Testosterone (T) and corticosterone (CORT) are critical hormones for normal development, differentiation, and adult function in both males and females. T is a steroid hormone that typically is higher in concentration in males than in females. This hormone has been shown to influence reproductive and aggressive behavior in both males and females. CORT is a glucocorticoid that is produced under stressful conditions,
when energy mobilization is necessary. Stress can result from typical (e.g., migration, over-wintering) as well as atypical (e.g., experimenter induced) events experienced by the individual. Glucocorticoids often vary inversely with reproductive steroids (e.g., Moore et al. 1991); extreme stressors must be overcome before energy allocation to breeding will occur. After Schwabl (1993) demonstrated that steroids such as T are deposited into yolk and can influence offspring phenotype in canaries and zebra finches, research into maternal effects via steroid transfer from mother to egg greatly expanded. Oviparous species became widely used for such studies after it was documented that steroids were found in yolks of eggs of not only birds, but also reptiles and, most likely, all types of eggs. Eggs of turtles (Janzen et al. 1998), sharks (Manire et al. 2004), fishes (McCormick 1998), alligators (Conley et al. 1997) and lizards (Lovern and Wade 2001) have been shown to contain steroid hormones.

Oviparous species are excellent models for maternal effects studies because after an egg is laid, no further transfer or chemical communication exists between the mother and the embryo, unlike placental species. Thus, the egg is a natural experimental package in which it is possible to determine and manipulate the embryo’s exposure to various compounds. For example, Adkins-Regan et al. (1995) found that estradiol injections into female Japanese quail resulted in elevated yolk estradiol concentrations, which caused an increased incidence of development of the right oviduct in adult offspring. Janczak et al. (2007) showed that increased yolk concentrations of CORT (accomplished by injecting eggs) did not affect chick tonic immobility in chickens, but did have the tendency to affect chick willingness to compete for food. Uller et al. (2007) demonstrated that lizard (Ctenophorus fordi) egg yolk T concentrations can be increased by injecting T directly
into the eggs and that this injected T causes an increase in growth rates among the hatchlings. Studies such as these add support to the idea that yolk steroids are not only present but also consequential to offspring phenotype. However, these steroids may have positive or negative influences on offspring phenotype. For example, Sockman and Schwabl (2000) showed that increased yolk T concentrations in American kestrels caused delayed hatching and reduced nestling growth and survival rates. On the other hand, Navara et al. (2006) concluded that elevated yolk T concentrations stimulate both early growth and immunity in developing house finches.

Current studies have also begun to manipulate the mother’s physical environment through dietary and light cycle changes, to determine how (or if) these changes affect steroid deposition. Warner et al. (2007) demonstrated that female lizards on poor-quality diets produced fewer clutches and deposited lower levels of T into their egg yolks. Lovern and Adams (2008) showed that female green anoles on enhanced diets had better body condition, produced more eggs, and had higher concentrations of T in their plasma than did females on a poor-quality diet, and that females on enhanced diets tended to increase their yolk T deposition with successive eggs.

In addition to direct changes in maternal endocrine status and physical condition, maternal social environment can influence yolk steroid deposition as well. And, just as reviewed above, studies that have looked at manipulating female social environment have yielded contradicting results (Table 1.1). For example, Marshall et al. (2005) did not demonstrate a significant difference in yolk T concentrations when female canaries were exposed to the songs of different males, but Tanvez et al. (2004) did demonstrate a significant difference in yolk androgen concentrations under similar experimental
conditions in canaries. It is apparent that more studies, incorporating a wider variety of study organisms, need to be conducted in order to develop an overall framework for understanding these and similar, seemingly contradictory, results.

**STUDY ORGANISM**

The green anole lizard (*Anolis carolinensis*) possesses genotypic sex determination (GSD) and has a well-studied natural history. *Anolis* encompasses approximately 400 species, in which *A. carolinensis* is the most studied species (Lovern et al. 2004). Green anoles have been used extensively in behavioral and ecological research for at least the past 100 years. Although most *Anolis* species are native to Central and South America, the green anole is native to North America and is common in the southeastern United States.

Green anoles are insectivorous, small lizards (typically < 70 mm snout-vent length) with a breeding season occurring approximately from April through July (Ruby 1984, Jenssen et al. 1995), although females may continue to lay eggs even after breeding has ended, using stored sperm (Conner and Crews 1980). Adult males establish large territories early in the breeding season and then aggressively defend them against other males, including non-territorial “floating” males. Male territories overlap multiple, smaller territories of adult females, but females do not establish their territories based on male location (Jenssen et al. 2001). Larger males tend to have larger territories, encompassing more female territories (Jenssen and Nunez 1998). Female home ranges tend to overlap, which results in an interesting social environment. Social behavior
among groups of females is significantly less aggressive than males, but agonism is still present. For example, females housed in groups in a laboratory establish a dominance hierarchy in which the dominant female may attack and bite subordinate females. Andrews and Summers (1996) discovered that even with an established dominance hierarchy, no difference in prey capture latency or success was found between dominant and subordinate females, but dominant females were more receptive to males.

In oviparous species, anything that a mother can provision to her eggs (e.g., additional yolk or yolk constituents such as lipids, antioxidants, or steroids) that would benefit her reproductive success and/or the potential reproductive success of her offspring has the potential to confer selective advantages. The social and reproductive framework of green anoles is, in many respects, suited for maternal effects research because green anoles do not provide parental care after egg-laying, females ovulate a single follicle at a time, yolk formation occurs in eggs independently, and single-egg clutches are laid approximately every 7-21 days (Crews 1980, Andrews 1985). These traits allow us to analyze sequential eggs during a single breeding season and provide us with the opportunity to evaluate any changes in allocation of steroid concentrations that may occur within the laying sequence, between treatments, and between alternations of the ovaries.

**OBJECTIVES**

I addressed two objectives in my thesis research project (Chapter II). The first was to determine how breeding density and year affect plasma and yolk T and CORT concentrations in a laboratory environment. I chose T as a steroid of interest because
several previous studies have evaluated yolk T levels in birds and I wanted to compare my results with previous results. Also, I chose CORT as a steroid of interest because few studies have evaluated yolk CORT concentrations. A change in social environment was expected to most likely affect T and CORT concentrations. To meet this objective, I housed male and female green anoles in one of two treatment groups: (1) low density (one male and one female) or (2) high density (one male and four females) for ten weeks over the course of two breeding seasons (2007 and 2008). I then measured the plasma and yolk T and CORT concentrations in males and females and in collected eggs using radioimmunoassay. I also measured egg mass, egg output, and body condition for males and females. The second objective was to document female plasma and yolk T and CORT concentrations in a field environment to determine how field data compare with laboratory data. To accomplish this objective, I collected blood samples and oviductal eggs from wild-caught females in Tyler, Texas. Once again, radioimmunoassay was used to measure plasma and yolk T and CORT concentrations, and once again I also measured egg mass and female body condition.
REFERENCES


Table 1.1. Results of literature search for studies that directly manipulated females either physically or socially to determine how maternal environment influences yolk hormone concentrations. The hormones examined in these studies were androgens, corticosterone (CORT), cortisol, dihydrotestosterone (DHT), estradiol (E$_2$), progesterone (P), prolactin (PRL), testosterone (T), and thyroxine (T$_4$). Physical manipulation studies either injected females with hormones or changed female diet, light cycle, etc. Social manipulation studies exposed females to varying breeding densities, looked at social status relationships, or exposed females to varying songs of males. Search conducted through Web of Science over the range of January 1993 – February 2009. Thirteen out of sixteen studies found an effect of physical aspects of maternal environment on yolk hormone concentrations. Eight out of ten studies found an effect of social aspects of maternal environment on yolk hormone concentrations.
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CHAPTER II

THE EFFECTS OF BREEDING DENSITY, YEAR, AND LABORATORY VS. FIELD ENVIRONMENTS ON PLASMA AND YOLK STEROIDS IN GREEN ANOLE LIZARDS (*Anolis carolinensis*)

ABSTRACT

Previous studies have demonstrated myriad effects of the social environment on reproductive physiology, and often these effects are communicated from mother to offspring via hormone exposure. The objectives of the present study were to: 1) determine how breeding density and year affect reproductive output and plasma and yolk testosterone (T) and corticosterone (CORT) concentrations in a laboratory environment; and 2) document female plasma and yolk T and CORT concentrations in a field environment for comparison to laboratory results. In the laboratory, egg mass and egg output were affected by year, but only output was affected by breeding density; females laid heavier and more eggs in 2008 than in 2007, and in both years females under low breeding density conditions laid more eggs than did females under high breeding density conditions. Female and male plasma T and CORT concentrations were unaffected by breeding density or year. In contrast, yolk T and CORT concentrations were affected by year but not by breeding density. Yolk T and CORT were both higher in 2007 than in
2008. Numerous correlations existed between male body condition and plasma steroids and the plasma and yolk steroids of females and eggs from within the same enclosure. For example, yolk CORT concentrations were highly negatively correlated with male plasma CORT concentrations. Finally, females sampled in the field, as compared to laboratory females, did not differ in plasma T concentrations but had lower plasma CORT concentrations. Both yolk T and CORT were significantly higher and more variable in eggs collected from females in the field. These results suggest that breeding density per se has little effect on plasma and yolk steroids in green anoles but that who you are breeding with can influence these parameters, that year-to-year variation in conditions may influence reproductive endocrinology and output, and that the factors influencing yolk steroid deposition are more numerous or stronger in the field than in the laboratory.

INTRODUCTION

Maternal effects may result from resources acquired in development, timing and location of birth or hatching, or behavior transmitted from mother to offspring (Qvarnström and Price 2001). Since the discovery of maternally-derived steroids in egg yolks, researchers have investigated this phenomenon as a potential tool for mothers to influence offspring phenotype. Although most maternal effects studies have used birds as models, yolk steroids can be found in a wide variety of animals including turtles (Janzen et al. 1998), sharks (Manire et al. 2004), fishes (McCormick 1998), alligators (Conley et
al. 1997) and lizards (Lovern and Wade 2001). Research on maternal effects can be conducted to assess effects on offspring by directly injecting eggs with steroids, or by manipulating the physical or social environment of females to determine how variation in these parameters influence yolk steroid deposition.

The majority of maternal effects studies have evaluated how the physical environment affects yolk steroid deposition. For example, in 1993, Ayson et al. discovered that yolk thyroxine concentrations increase as female rabbitfish are injected with thyroxine and Warner et al. (2007) showed that jacky dragon maternal nutrition alters yolk testosterone (T) concentrations (females on poor-quality diets laid eggs with lower T concentrations). On the other hand, Salvante et al. (2003) did not observe changes in yolk corticosterone (CORT) when female zebra finches were injected with CORT. Of the few studies that have researched how a female’s social environment affects yolk steroid deposition, results among these studies greatly differ. For example, Navara et al. (2006) demonstrated that intruder presentations to female Eastern bluebirds caused higher yolk androgen concentrations to be deposited into their eggs, but Marshall et al. (2005) showed that female canaries did not alter yolk T concentrations when exposed to different qualities of male song. Across species, some features of the social environment apparently are salient with respect to steroid deposition, whereas others are not.

The green anole (*Anolis carolinensis*) is an excellent model for investigating the effects of maternal steroids on offspring phenotype because we know much about its biology through a long history of evolutionary, ecological, physiological, and behavioral studies (e.g., Crews 1980; Lovern et al. 2004). Because green anoles lay single-egg
clutches during the breeding season, with the left and right ovary typically alternating in egg production, processes in egg formation are independent for each offspring produced and changes in maternal condition have the potential to influence individual offspring phenotypes. Therefore, the social environment of breeding females has the potential to affect steroid production, and this could be demonstrated by differences found in circulating plasma and yolk steroid levels. Two studies have already examined how the physical environment of female green anoles affects yolk steroid deposition. Walguarnery and Lovern (2008) found that females housed in larger cages had higher concentrations of plasma T and deposited more estradiol into their eggs than females housed in smaller cages, although no differences were seen in yolk T or CORT. Lovern and Adams (2008) found that females given enhanced quality diets had higher plasma T concentrations and deposit increasing yolk T concentrations with successively laid eggs, as compared to females on standard or reduced quality diets. There were no treatment effects on yolk CORT. These studies demonstrate that the physical environment has the potential to affect female reproductive endocrinology and yolk steroid deposition in green anoles.

In the present study, I investigated the relationship between social conditions and plasma and yolk steroids in green anoles. Specifically, for objective one I documented effects of breeding density on plasma and yolk T and CORT in a laboratory setting by housing males and females in one of two treatments: 1) one male and one female per cage (low density); or 2) one male and four females per cage (high density). I also examined the plasma T and CORT concentrations of the resident males, body condition of males and females, egg mass, and egg output. For objective two, I documented female plasma
and yolk T and CORT concentrations in a field environment for comparison to results obtained in the laboratory.

METHODS

Laboratory component

I purchased reproductively active adult green anole (A. carolinensis) females and males from Charles Sullivan Company (Nashville, TN) in June 2007 and Candy’s Quality Reptiles (La Place, LA) in May 2008; both suppliers collect their lizards from Louisiana. Lizards were housed in thirty-one 110-L glass aquaria in the laboratory. Each cage consisted of a peat moss substrate, water dish, 0.5-L plastic nest box (filled with dampened peat moss and lids with small openings to allow females to enter and deposit eggs), screen cage top, and ultraviolet (Repti-Sun, Zoo Med, Inc.) and incandescent (60 watt, Phillips) lighting for UVB exposure and basking, respectively. Rocks and dowels were also placed in the cages to serve as basking and hiding spots for the lizards.

All lights in the laboratory were set on timers to simulate natural breeding conditions (14:10 light:dark cycle for the overhead fluorescent lights, 12:12 light:dark cycle for the cagetop ultraviolet lights, and 10:14 light:dark cycle for the cagetop incandescent lights). The room temperature was 25-28°C, with daytime temperatures ranging between 22-38°C inside the cages, depending on location in each cage with respect to the basking light. Relative humidity averaged 65%.
Upon arrival into the laboratory, I clipped the green anoles’ toes for individual identification and randomly assigned each anole to one of two treatment groups: (1) low density (one male and one female per cage; N=14 cages [4 in 2007 and 10 in 2008]) or (2) high density (one male and four females per cage; N=17 cages [5 in 2007 and 12 in 2008]). I then measured males and females for their snout-vent length (SVL) to the nearest mm and mass to the nearest 0.01 g. Lizards were fed every other day with crickets or mealworms (2007) or crickets, mealworms, or wax worms (2008). At alternate feedings of crickets, I dusted food items with vitamin and mineral supplement powders (Herptivite\textsuperscript{TM} from Rep-Cal, Los Gatos, CA, and Miner-All\textsuperscript{TM} from Sticky Tongue Farms, Sun City, CA). The number of prey items put into each cage was proportional to the number of lizards in that cage, so that individuals in all cages had equal access to food. On a daily basis, I misted cages with water and checked nest boxes for eggs. Egg mass was recorded to the nearest mg and all eggs were placed into separate, individually labeled containers and stored at -20°C until analysis for steroid content. I collected 41 eggs in 2007 and 158 eggs in 2008.

In both 2007 and 2008, at the end of ten weeks I again recorded each individual’s SVL to the nearest mm and mass to the nearest 0.01 g. Individual blood samples were collected from the trunk following decapitation within five minutes of capture. I collected the samples between 0900-1200 to minimize sample variation due to normal fluctuating steroid concentrations throughout the day. Blood samples were centrifuged and the plasma was stored at -20°C until analysis for steroid content.
Field component

Seven reproductively active adult female green anoles that had shelled eggs (determined by palpation) were collected from Camp Tyler in Tyler, Texas, on 20 June 2008. Female SVL and mass were recorded to the nearest mm and 0.01 g, respectively. Individual blood samples were taken from each female from the trunk, following decapitation, within five minutes of capture. The blood samples were collected between 0900-1200. Following blood collection, the abdominal cavity of each female was opened and the shelled egg was removed. Egg mass was recorded to the nearest mg for each egg and then the eggs were placed in separate, individually labeled containers. Blood samples and eggs were placed in a portable cooler with ice while in the field. All blood samples were centrifuged and the plasma was removed and stored, along with eggs, frozen in a cooler containing dry ice for return to the laboratory. Once in the laboratory, I placed all eggs and plasma samples in a freezer at -20°C until analysis for steroid content.

Radioimmunoassay

I analyzed all laboratory and field yolk and plasma samples for testosterone (T) and corticosterone (CORT) by radioimmunoassay (RIA) following extraction and chromatographic separation (Wingfield and Farner 1975; Schwabl 1993). Samples were thawed and diluted with ddH2O to create a sufficient volume for extraction. Tritiated T and CORT (PerkinElmer Life Sciences, Inc.) were added to each sample and samples were kept in a refrigerator overnight. The next day, each sample was extracted twice with
diethyl ether (plasma) or 70:30 diethyl ether:petroleum ether (yolk), and then dried in a 37 °C water bath and with nitrogen gas. This completed the extraction procedure for the plasma samples (see below). Yolk samples were reconstituted in 95% ethanol and stored at -20 °C overnight. The following day, they were centrifuged and the supernatant was transferred to clean test tubes and dried in a 37 °C water bath and with nitrogen gas. Following extraction, yolk and plasma samples were both reconstituted in 10% ethyl acetate in isooctane. Purification and separation were achieved via column chromatography. Once the T and CORT fractions were collected, the samples were dried under nitrogen gas and then resuspended in assay buffer and placed in a refrigerator overnight. Tritiated steroid tracers and antisera (T from Research Diagnostic Products and CORT from Sigma) were used the following day to complete competitive binding in the radioimmunoassay. Standard curves were run in triplicate and samples were run singly over the course of seven assays. Samples were arbitrarily assigned to assays and plasma and yolk samples were run separately. Plasma and yolk samples were also run separately by year. Average intra-assay coefficient of variation (CV) was 0.236 for T and 0.283 for CORT; inter-assay CV was 0.385 for T and 0.298 for CORT.

Statistical analyses

Body condition was defined as the residual of the regression of mass onto SVL (Schulte-Hostedde et al. 2005). I analyzed the data for treatment effects (breeding density), year effects (2007, 2008), and treatment/year interaction effects on female body condition, egg mass and number, and plasma and yolk T and CORT concentrations by
using general linear model (GLM) analyses of variance (ANOVA). All interaction
effects were either non-significant ($P > 0.10$) or marginally significant ($0.05 < P < 0.10$)
Because cages contained multiple individuals that could have an influence on the data
collected from individuals, data were analyzed by overall values, treating individuals as
independent samples, and by within-cage averages, treating cages as independent
samples. Spearman rank order correlation analyses were used to examine relationships
among male and female body condition, plasma steroids, egg mass, egg output, and yolk
steroids. Statistical analyses were run using Minitab 14 or SigmaStat 3.1; differences
were considered statistically significant when $P < 0.05$ and marginally significant when $P
< 0.10$.

RESULTS

Laboratory component-body condition, egg mass, and egg output

Treatment and year interaction effects for male body condition were non-
significant (initial body condition: $F_{1,28} = 0.03, P = 0.867$; final body condition: $F_{1,28} =
0.33, P = 0.571$; body condition change: $F_{1,28} = 0.44, P = 0.515$). Male initial body
condition entering the laboratory did not differ with treatment ($F_{1,28} = 0.22, P = 0.644$),
but it did differ with year; males in 2007 had lower initial body condition than males in
2008 ($F_{1,28} = 14.11, P = 0.001$). There were marginally significant treatment effects and
significant year effects for male final body condition and change in body condition.
Males from cages with low breeding densities tended to have lower final body condition and a sharper decline in body condition over the course of the treatment than did males from cages with high breeding densities (final body condition: \( F_{1,28} = 3.20, P = 0.086; \) body condition change: \( F_{1,28} = 4.08, P = 0.054; \) Fig. 1A). Additionally, males in 2007 had lower final body condition and a sharper decline in body condition than males in 2008 (final body condition: \( F_{1,28} = 26.37, P < 0.0005; \) body condition change: \( F_{1,28} = 6.32, P = 0.019). Treatment and year interaction effects for female body condition were non-significant when examined by either individual female (initial body condition: \( F_{1,28} = 0.50, P = 0.481; \) final body condition: \( F_{1,28} = 0.14, P = 0.711; \) body condition change: \( F_{1,28} = 0.74, P = 0.393) \) or within-cage averages (initial body condition: \( F_{1,28} = 0.08, P = 0.785; \) final body condition: \( F_{1,28} = 1.30, P = 0.265; F_{1,28} = 0.74, P = 0.399) \). Female initial, final, and change in body condition all were unaffected by treatment when examined by individual female (initial body condition: \( F_{1,78} = 0.05, P = 0.824; \) final body condition: \( F_{1,78} = 0.02, P = 0.876; \) body condition change: \( F_{1,78} = 0.09, P = 0.760; \) Fig. 1B) as well as by within-cage averages for initial (\( F_{1,28} = 0.58, P = 0.454 \)) and final body condition (\( F_{1,28} = 2.74, P = 0.111 \)). When evaluating female body condition by within-cage averages, females in low density breeding groups increased in body condition over the course of the study when compared to females in high density breeding groups (\( F_{1,28} = 4.82, P = 0.038 \)). Similarly, year did not affect the final body condition of females (\( F_{1,78} = 0.02, P = 0.875 \)), but females in 2007 had lower initial body condition and a greater increase in body condition compared to females in 2008 (initial body condition: \( F_{1,78} = 11.25, P = 0.001; \) body condition change: \( F_{1,78} = 5.26, P = 0.025 \)); this was also true when examined by within-cage averages (final body condition: \( F_{1,28} = 0.04, P = 0.847; \)
initial body condition: $F_{1,28} = 12.93, P = 0.001$; body condition change: $F_{1,28} = 7.60, P = 0.011$). There was a significant positive correlation between the initial body condition of females and both the final body condition of males ($r = 0.530, P = 0.003$) and male change in body condition ($r = 0.479, P = 0.009$).

Treatment and year interaction effects were non-significant for egg mass when evaluated by individual eggs ($F_{1,198} = 1.24, P = 0.267$) or within-cage averages ($F_{1,32} = 0.47, P = 0.496$). Egg mass was unaffected by treatment both overall ($F_{1,198} = 0.06, P = 0.812$) and when examined by within-cage averages ($F_{1,32} = 0.38, P = 0.541$). However, eggs were significantly heavier in 2008 than in 2007 overall ($F_{1,198} = 5.43, P = 0.021$; Fig. 2A) and tended to be heavier in 2008 than 2007 when examined by cage ($F_{1,32} = 3.55, P = 0.070$). Treatment and year interaction effects were marginally significant for egg output per female ($F_{1,29} = 3.84, P = 0.061$). Egg output per female was significantly affected by treatment and year (Fig. 2B). Females in cages with low breeding densities produced more eggs than females in cages with high breeding densities (egg output per female: $F_{1,29} = 19.03, P < 0.0005$). Females in 2008 produced more eggs than females in 2007 ($F_{1,29} = 10.85, P = 0.003$). Finally, there was a significant positive correlation between the average of within-cage egg masses and the initial body condition of the male in that cage ($r = 0.422, P = 0.023$). No significant correlations existed between egg mass and female body condition, male final body condition, male change in body condition, or egg output (all $P > 0.05$).
Laboratory component—plasma and yolk testosterone (T) and corticosterone (CORT) concentrations

Treatment and year interaction effects were non-significant for male plasma T and CORT concentrations (plasma T: $F_{1,28} = 1.16$, $P = 0.292$; plasma CORT: $F_{1,28} = 1.62$, $P = 0.214$). Males in high density cages tended to have higher T concentrations than males in low density cages ($F_{1,28} = 3.60$, $P = 0.069$), but year did not affect male plasma T ($F_{1,28} < 0.0005$, $P = 0.959$). Male plasma CORT was not affected by treatment or year (breeding density: $F_{1,28} = 0.03$, $P = 0.854$; year: $F_{1,28} = 2.53$, $P = 0.124$; Fig. 3A). Treatment and year interaction effects were non-significant for female plasma T and CORT concentrations when evaluated by individual female (plasma T: $F_{1,78} = 0.66$, $P = 0.420$; plasma CORT: $F_{1,78} = 1.41$, $P = 0.238$) or within-cage averages (plasma T: $F_{1,28} = 0.41$, $P = 0.529$; plasma CORT: $F_{1,28} < 0.0005$, $P = 0.974$). There were no treatment or year effects on female plasma T (breeding density: $F_{1,78} = 0.08$, $P = 0.776$; year: $F_{1,78} = 0.01$, $P = 0.934$) when analyzing individual females. No difference was seen in plasma CORT levels between treatments ($F_{1,78} = 1.22$, $P = 0.273$), but females in 2008 had higher plasma CORT concentrations than females in 2007 ($F_{1,78} = 4.13$, $P = 0.046$; Fig. 3B) when analyzing individual females. The analysis of female plasma T and CORT using within-cage averages produced similar results; breeding density did not affect female plasma T ($F_{1,28} = 1.25$, $P = 0.274$) or female plasma CORT ($F_{1,28} = 0.02$, $P = 0.893$) and year did not affect female plasma T ($F_{1,28} = 0.03$, $P = 0.857$), but females in 2008 had significantly higher concentrations of plasma CORT than did females in 2007 ($F_{1,28} = 5.88$, $P = 0.023$).
Treatment and year interaction effects were non-significant for yolk T concentrations, but were marginally significant for yolk CORT concentrations when analyzed by individual eggs (yolk T: $F_{1,198} = 1.66, P = 0.199$; yolk CORT: $F_{1,198} = 3.20, P = 0.075$) and within-cage averages (yolk T: $F_{1,32} = 0.22, P = 0.641$; yolk CORT: $F_{1,32} = 2.96, P = 0.098$). Yolk T was unaffected by breeding density for individual eggs ($F_{1,198} = 0.38, P = 0.541$; Fig. 4A) and for within-cage averages ($F_{1,32} < 0.0005, P = 0.962$), but yolk T was significantly higher in 2007 than 2008 for individual eggs ($F_{1,198} = 6.56, P = 0.011$) and for within-cage averages ($F_{1,32} = 7.49, P = 0.011$). Yolk CORT was marginally affected by breeding density ($F_{1,198} = 3.42, P = 0.066$; Fig. 4B) and significantly affected by year ($F_{1,198} = 9.83, P = 0.002$) when analyzed by individual eggs. Similarly, yolk CORT was not affected by treatment ($F_{1,32} = 1.19, P = 0.286$), but yolk CORT was significantly higher in 2007 than 2008 ($F_{1,32} = 12.67, P = 0.002$) when analyzed by within-cage averages.

Numerous correlations existed between body condition and steroid concentrations. Female final body condition and change in body condition negatively correlated with plasma T concentration (final body condition: $r = -0.239, P = 0.034$; change in body condition: $r = -0.236, P = 0.037$). There were significant positive correlations between female plasma CORT concentration and female plasma T concentration ($r = 0.294, P = 0.009$), male plasma T concentration ($r = 0.464, P = 0.012$), male final body condition ($r = 0.470, P = 0.010$), and male change in body condition ($r = 0.369, P = 0.048$). Yolk CORT concentrations were highly negatively correlated with male plasma CORT concentrations ($r = -0.599, P < 0.0005$) and egg mass ($r = -0.483, P = 0.008$), and marginally negatively correlated with male initial body condition ($r = -$
0.360, \( P = 0.055 \), but marginally positively correlated with male plasma T concentrations \((r = 0.314, \ P = 0.097)\) and positively correlated with yolk T concentrations \((r = 0.391, \ P = 0.036)\). No other possible correlations existed among male body condition, female body condition, egg mass, egg output, male plasma T, male plasma CORT, female plasma T, female plasma CORT, yolk T, and yolk CORT (all \( P > 0.10 \)).

*Comparisons between laboratory and field data*

Laboratory data were combined across year and treatment for comparison to body condition and plasma and yolk steroids of field-sampled females. Field-sampled females had higher SVL and mass than females in the laboratory (SVL: \( F_{1,85} = 34.87, \ P < 0.0005 \); mass: \( F_{1,85} = 26.95, \ P < 0.0005 \), but body condition did not differ \((F_{1,85} = 0.57, \ P = 0.453)\). Plasma T also did not differ between laboratory and field-sampled females \((F_{1,85} = 0.22, \ P = 0.640)\), but females housed in the laboratory had higher concentrations of plasma CORT than did females sampled in the field \((F_{1,85} = 4.66, \ P = 0.034; \text{Table 2.1})\). Eggs from laboratory females were heavier than eggs collected from females in the wild, whether examined for individual eggs \((F_{1,205} = 4.28, \ P = 0.040)\) or by within-cage averages \((F_{1,39} = 6.53, \ P = 0.015)\). Both yolk T and yolk CORT concentrations were higher in eggs from field females than eggs from females in the laboratory \((\text{yolk T: } F_{1,205} = 192.36, \ P < 0.0005; \text{yolk CORT: } F_{1,205} = 15.93, \ P < 0.0005; \text{Table 2.1})\). Yolk T and yolk CORT concentrations from the laboratory and field had variances that significantly
differed when analyzed with an F-test (yolk T: $F = 116, P < 0.0005$; yolk CORT: $F = 7.88, P < 0.0005$).

**DISCUSSION**

In this study I have demonstrated that annual variation can exist in reproductive output, endocrinology, and individual response to the social variable of breeding density as manipulated in the laboratory (e.g., changes in body condition). Breeding density had a strong effect on egg output, with females under low breeding density conditions producing more eggs than females under high breeding density conditions over the course of ten weeks in the laboratory even though food was allocated proportionally; other reproductive and endocrine parameters were largely unaffected by breeding density per se. In contrast, year (2007 vs. 2008) had widespread effects on body condition (higher average body condition in 2008 for males with a net increase in body condition over the course of the study, higher average body condition for females in 2008 with a net decrease in body condition over the course of the study), egg mass and output (higher in 2008), female plasma CORT (higher in 2008) and yolk T and CORT (lower in 2008). Additionally, male traits including body condition and plasma T and CORT showed associations with female traits including body condition, egg mass, and plasma and yolk steroids. Overall, these results suggest that although females do not substantially alter yolk steroid concentrations in response to breeding density, they do alter reproductive allocation in terms of egg number and yolk T and CORT deposition in relation to traits of the male territory-holder, regardless of breeding density. In comparison to laboratory-
generated data, field-sampled females had lower plasma CORT and produced eggs with less mass, but containing higher and more variable concentrations of yolk T and CORT. These results suggest that females in the field and laboratory are exposed to a different range of parameters that influence yolk steroid deposition and that those parameters are both more varied and variable in the field.

*Laboratory component*

Year differences in initial body condition for males and females showed how using different animals each year causes individual variability to become more obvious. All males declined in body condition in 2007, but only low density males declined in body condition in 2008. Male body condition loss may be due to the physical strain of courtship displays and mating. Males in high density cages had access to more resources such as food, which may explain the 2008 differences between treatments. In 2007, all females gained body condition, but in 2008 females in high density cages lost body condition, whereas females in low density cages had no effect on body condition. This is in spite of the fact that in 2008 females produced more eggs on average. Feeding differences between years may partially account for this difference; although fed the same number of times per week, lizards in 2008, but not 2007, received waxworms once per week in addition to crickets and mealworms. However, this would not account for the initial difference in body condition that we saw in which lizards entered the lab in better body condition in 2008 vs. 2007.
Plasma testosterone (T) and corticosterone (CORT) were unaffected by year or treatment for both males and females, with a few exceptions. Male plasma T tended to be higher in males from high breeding densities than males from low breeding densities. Males in high breeding densities may have higher T levels due to additional effort put towards courtship and mating with multiple females. Also, female plasma CORT concentrations were higher in 2008 than 2007. The relationship between T and CORT has been previously viewed as a negative correlation in green anoles, but this study along with Lovern and Adams (2008) suggests that plasma and yolk T and CORT concentrations are positively correlated. Plasma CORT levels in this study did not inhibit breeding in this study, which offers the idea that perhaps only extreme (outside the range of this study) CORT levels may hinder breeding in the green anole. Yolk T was unaffected by treatment and yolk CORT was marginally higher in eggs from high breeding densities, but both yolk T and yolk CORT were higher in 2007 than 2008. These results were not consistent with the results of the majority of studies that have manipulated social conditions and evaluated yolk T concentrations. For example, Reed et al. (2001), Müller et al. (2002), Michl et al. (2004), Tanvez et al. (2004), and Navara et al. (2006) found changes in yolk T levels, whereas Marshall et al. (2005) did not find changes in yolk T levels. Unfortunately, it appears that no previous studies have manipulated social conditions and evaluated yolk CORT concentrations.

For *A. carolinensis*, a variety of potential physical and social influences on plasma and yolk steroid concentrations have now been explored. In reproductively active females, plasma steroid concentrations appear to be influenced in the laboratory by cage size (higher T and lower CORT in large cages; Walguarnery and Lovern 2008) and diet
(higher T with enhanced diet quality; Lovern and Adams 2008), but not cage density (this study). Yolk steroid concentrations--which have been shown to vary substantially among females (e.g., Lovern and Adams 2008)--are, in contrast, unrelated to the potential treatment effects examined thus far (except for yolk estradiol, which was higher in large cages; Walguarnery and Lovern 2008). For green anoles, when resources or conditions vary, the primary reproductive decision made by females appears to be how frequently to produce eggs, not how big to make them or what to put in them.

One important exception to this trend may be associations of male body condition and reproductive endocrinology with female reproductive endocrinology and output. I found that indices of male body condition associated with female plasma CORT concentrations, egg mass, and yolk CORT concentrations. Previous studies on male influence on female reproduction have found similar relationships. For example, Marshall et al. (2005) discovered that high-quality male songs resulted in higher female plasma T levels, but no difference in yolk T levels for canaries. In comparison, Tanvez et al. (2004) concluded that high-quality male songs resulted in higher yolk androgen levels in canaries. Also, Kingma et al. (2009) found that when female blue tits were exposed to males with higher-quality UV coloration in their crown feathers, an increase in yolk androgen concentrations occurred. These studies suggest that more research in this area is essential to acquire a better understanding of the relationship between male reproductive quality and female and yolk T and CORT concentrations.
Field component

Although one expects the laboratory and field environments to be different--often by design--the magnitude of the yolk steroid differences between eggs collected from females housed in the laboratory and those collected directly from females sampled in the field was surprising. Yolk T and CORT concentrations were higher in eggs collected in the field by fifteen-fold and nearly four-fold, respectively; variation (SE) was over 40-fold higher for yolk T and 15-fold higher for yolk CORT from field eggs. This is in spite of the fact that body condition did not differ between field and laboratory females (although field females were larger and heavier on average), that plasma T did not differ, and that plasma CORT was actually higher in laboratory-housed females (consistent with Walguarnery & Lovern 2008). Additionally, eggs collected from females in the field were lighter than eggs collected from females housed in the laboratory (258 ± 21 mg vs. 286 ± 3 mg; 11% difference). While this could be due to field vs. laboratory differences in food intake or some other experimental effect, it also could be the result of methodology; eggs in the laboratory were collected following oviposition and by necessity in the field they were collected directly from the oviduct, potentially prior to additional shelling and/or water uptake that would have contributed mass.

Currently, it appears that this study is one of the first to compare measurements of yolk steroids in the laboratory and field. Differences between laboratory and field values could have resulted from greatly simplified inputs in the laboratory compared to the field. In the field, individuals are exposed to the full range of potential environmental and social influences simultaneously rather than in near-isolation, as intended in the
laboratory. These results should be confirmed with a larger field sample, but thus far the message is that, as always, we need to be cautious in both design and interpretation of laboratory studies when trying to compare to the natural world and its full range of inputs as experienced in the field.

ACKNOWLEDGMENTS

I thank Jondrea Chesser, Angie Reisch, Michelle Sargent, and Chelsea Williams for help with lizard care. I am grateful for Alan Byboth for allowing me to collect anoles on the Camp Tyler premises. This research was supported by NSF grant IOS-0641434 to MBL.
REFERENCES


FIGURE LEGENDS

Fig. 1. Mean (± 1 SE) change in body condition from the beginning to the end of the study for individual laboratory males (A) and females (B), where body condition is defined as the residual of the regression of mass on SVL. High density values are shown with black bars and low density values are shown with gray bars.

Fig. 2. Mean (± 1 SE) individual egg mass (A) and egg output per female (within-cage average; B) in low and high breeding densities in the laboratory. High density values are shown with black bars and low density values are shown with gray bars.

Fig. 3. Mean (± 1 SE) plasma T and CORT concentrations for individual laboratory males (A) and females (B). High density T values are shown with black bars, low density T values are shown with medium gray bars, high density CORT values are shown with dark gray bars, and low density CORT values are shown with light gray bars.

Fig. 4. Mean (± 1 SE) yolk T (A) and yolk CORT (B) concentrations for individual laboratory yolk samples. High density values are shown with black bars and low density values are shown with gray bars.
Year | Change in Body Condition
--- | ---
2007 | -0.8
2008 | -0.6
2009 | -0.4
2010 | -0.2
2011 | 0.0
2012 | 0.2
2013 | 0.4
2014 | 0.6
2015 | 0.8

A

Year | Male Change in Body Condition
--- | ---
2007 | -0.2
2008 | 0.0

B

Year | Female Change in Body Condition
--- | ---
2007 | 0.2
2008 | 0.4
### Egg Mass (mg)

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### Total Eggs (per female)

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<th>Year</th>
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TABLE LEGENDS

Table 2.1. Comparisons between laboratory and field values for mean ± 1 SE female plasma and yolk T and CORT concentrations. Sample size for field females equaled seven.
<table>
<thead>
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<th></th>
<th>Laboratory</th>
<th>Field</th>
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<tr>
<td>Mean Female Plasma T (ng/ml)</td>
<td>0.521±0.080</td>
<td>0.393±0.149</td>
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<td>Mean Female Plasma CORT</td>
<td>20.650±1.170</td>
<td>12.110±1.390</td>
<td>4.66</td>
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<td>0.034</td>
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<td>Mean Yolk T (pg/mg)</td>
<td>0.783±0.073</td>
<td>12.270±4.190</td>
<td>192.36</td>
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<tr>
<td>Mean Yolk CORT (pg/mg)</td>
<td>0.7418±0.0841</td>
<td>2.740±1.260</td>
<td>15.93</td>
<td>1, 205</td>
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</tr>
</tbody>
</table>
VITA

Amber Lynn Adams

Candidate for the Degree of

Master of Science

Thesis: THE EFFECTS OF BREEDING DENSITY, YEAR, AND LABORATORY VS. FIELD ENVIRONMENTS ON PLASMA AND YOLK STEROIDS IN GREEN ANOLE LIZARDS (Anolis carolinensis)

Major Field: Zoology

Biographical:

Personal Data: Born in Stevens Point, Wisconsin, on March 7, 1985, the daughter of Larry and Mary Adams and granddaughter of Bernice Nystrom and Carl and Dorothy Adams.

Education: Graduated from Tri-County High School, Plainfield, Wisconsin, in May 2003. Received Bachelor of Science degree with a major in Animal Science at the University of Wisconsin-River Falls, River Falls, Wisconsin, in May 2007. Completed the requirements for the Master of Science in Zoology at Oklahoma State University, Stillwater, Oklahoma, in May 2009.

Experience: Employed as assistant in the Physiology Laboratory, Animal Science Department, University of Wisconsin-River Falls, August 2003 to May 2004; Employed as animal science tutor, University of Wisconsin-River Falls, August 2005 to August 2006; Served as research assistant in the Department of Zoology at Oklahoma State University, June 2007 to August 2008; Served as teaching assistant in the Department of Zoology at Oklahoma State University, August 2008 to May 2009.

Professional Memberships: American Rabbit Breeders Association, Oklahoma State University Zoology Graduate Student Society, Society for Integrative and Comparative Biology
Title of Study: THE EFFECTS OF BREEDING DENSITY, YEAR, AND LABORATORY VS. FIELD ENVIRONMENTS ON PLASMA AND YOLK STEROIDS IN GREEN ANOLE LIZARDS (Anolis carolinensis)

Scope and Method of Study: My objectives were to: 1) determine how breeding density and year affect plasma and yolk T and CORT concentrations in a laboratory environment; and 2) document female plasma and yolk T and CORT concentrations in a field environment. Breeding females were housed in cages with either one male (low density) or one male and three additional females (high density) to evaluate the effects of breeding density on reproductive effort and plasma and yolk testosterone (T) and corticosterone (CORT) concentrations. All laboratory animals were maintained under standard breeding conditions for ten weeks over the course of two breeding seasons. Nest boxes were checked daily for eggs which were frozen for subsequent yolk steroid analyses. At the end of the study, blood samples were collected from all laboratory animals. Blood samples and eggs were collected from wild-caught females. All blood and yolk samples were analyzed using radioimmunoassay.

Findings and Conclusions: I found that breeding density did not affect male initial body condition, but did affect male final body condition and change in body condition. A significant year effect was seen in male initial, final, and change in body condition. Breeding density did not affect female body condition, but year effects were seen in female initial and change in body condition. Egg mass was affected by year, but not breeding density. Surprisingly, females in low density cages produced more eggs than females in high density cages and year also affected egg production. Female and male plasma CORT and T concentrations were unaffected by breeding density or year, except males in high breeding densities tended to have higher plasma T concentrations and females in 2008 had higher plasma CORT concentrations than females in 2007. Yolk T and CORT concentrations were affected by year and yolk CORT concentrations tended to be higher in eggs from high breeding densities, rather than low breeding densities. In comparison with field data, eggs from females in the laboratory were heavier, but had lower levels of yolk T and CORT. Wild-caught females tended to have greater mass and SVL than laboratory females, but laboratory females had higher concentrations of plasma CORT.