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The Reproductive Cycle of the Guatemalan Beaded Lizard, *Heloderma charlesbogerti*

Wade C. Carruth III

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THE REPRODUCTIVE CYCLE OF THE GUATEMALAN BEADED LIZARD,
HELODERMA CHARLESBOGERTI

by

WADE C. CARRUTH III

(Under the direction of David C. Rostal)

ABSTRACT

Reproductive information, including seasonality in behavior and physiology, is key to our ability to breed species for maintaining assurance colonies or for future introductions. Limited scientific data is available about the reproduction of Helodermatid lizards. Several species of helodermatid lizard are uncommon, rare, or simply exist at low population densities and in need of the development of conservation programs and management. Most helodermatid reproductive data collected to date has been done on deceased animals using histological techniques. Until this study, no hormones have been analyzed, and individual animals have not been followed through a complete cycle. The purpose of this study was to delineate the reproductive cycle of the Guatemalan beaded lizard, *Heloderma charlesbogerti*, by monitoring seasonal steroid and calcium cycles, vitellogenesis, ovarian follicular growth, and egg production. Blood samples were collected monthly from adult captive lizards housed at Zoo Atlanta to determine circulating hormone levels. Testosterone and corticosterone levels in males and estradiol, corticosterone and calcium levels in females, were correlated with female reproductive condition determined by ultrasonography. Testosterone in male lizards peaked during August indicating that breeding should occur in September-October. Consistent with what has been observed in deceased wild specimens of Mexican beaded lizards (*Heloderma horridum*) with spermatogenesis in August through October. A distinct ovarian cycle was observed with small previtellogenic follicles appearing as early as November and vitellogenesis occurring from May to November. Ovulatory

estradiol spikes were identified in some females in August and November. Corticosterone levels appear to increase in gravid females.

INDEX WORDS: Beaded lizard, Reproductive hormones, *Heloderma charlesbogerti*, captive reproduction, Helodermatidae, helodermatid

THE REPRODUCTIVE CYCLE OF THE GUATEMALAN BEADED LIZARD,
HELODERMA CHARLESBOGERTI

by

WADE C. CARRUTH III

B.S., Georgia Southern University, 2010

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial
Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE
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WADE C. CARRUTH III

Major Professor: David C. Rostal

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DEDICATION

To Michelle, the best person that I could have ever hoped with whom to spend the rest of my life.

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I would like to thank everyone who has helped me on this journey. The list of names is quite long, beginning with my parents Wade and Claire Carruth and sister Katherine. They have always supported me both financially and emotionally whenever I needed it, without which none of this would have been possible. To my wife Michelle, the best... well everything. I love her unconditionally, and I could not be a luckier man to have her in my life.

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CHAPTER 1

INTRODUCTION

Reproductive Physiology as a Tool in Conservation Biology

Most available lizard hormone data comes from the iguanid group, specifically the genera *Anolis* and *Sceloporus* (Lovern 2011). Research on reproductive biology and endocrinology of reptiles shows consistencies in seasonal steroid hormone cycles, breeding behavior and nesting activities. Thus, the roles of reproductive hormones remain conserved among reptilian species (Lovern 2011), this allows for comparisons among species, creating a database to learn about reptilian reproduction. In males, plasma testosterone levels are maximal during breeding (Kumar et al. 2011). Testosterone can also be found in females of several species at lower concentrations than males and varies significantly through the female reproductive cycle (Jones 2011). In females, progesterone rises after vitellogenesis and stays high during the reproductive season (Jones 2011). Estradiol is high during vitellogenesis and declines through the gravid stage, and can vary based on ovulatory cycle (ex. if they multi-clutch, McPherson et al. 1982) (Lovern 2011). Environmental cues such as increasing day length and temperature can vary based on the type ecosystem and are important for initiating these physiological changes.

Temperate zones have well defined seasons with respect to day length and temperature. Increasing day length triggers the pineal gland to produce melatonin, which starts a cascading effect, influencing the hypothalamus and then the gonads. Tropical zones have well defined seasons with respect to temperature and rainfall. Seasonal rainfall is then associated with food availability. While these zones have different environmental cues, these cues drive similar physiological changes in males and females, sometimes in different ways, which start breeding. Another consideration is what happens when you change an animal's location. Here we are

looking at beaded lizards that have been removed from their natural habitat (tropical) and are being kept in a captive environment (temperate). Now seasonal changes (cues) are not the same. We as animal keepers must attempt to emulate the wild conditions of these animals. Most reptiles appear to keep the cycle of their native range, as opposed to adjusting to their new environment. For example, leopard tortoises lay eggs in the warm months of May to June or November to December, depending on latitude (Bonin et al. 2006). Most captive animals in the United States still lay eggs from November to December, during the cold winter months.

Reproductive patterns of squamates were first generalized by Licht, Saint Girons, et al. (summarized in Licht 1984). Reproductive patterns were characterized by quantifying relationships between mating behavior, sex steroid production and gametogenesis. Two generic patterns occur: prenuptial (Type I) and postnuptial (Type II). These patterns were further generalized by Crews 1984 into associated, roughly equal to Type I, and dissociated, roughly equal to Type II. The associated reproductive pattern is when mating behavior, sex steroid production and gametogenesis happen at roughly the same time (Males: *Geochelone nigra*, *Podarcis s. sicula*, *Calotes versicolor*, *Sternotherus odoratus*). The dissociated pattern is when these happen at different times (Males: *Chrysemys picta*, *Chelydra serpentina*, *Chelonia mydas*). Crews (1984) has been highly influential in assessing the reproductive patterns of numerous species, including lizards. The large portion of lizard species display an associated reproductive pattern (Lovern 2011); however, other patterns may occur.

In order to establish successful captive reproductive programs, knowledge of the life-history of an organism is necessary. This is especially so when dealing with long-lived species of reptiles (Congdon et al. 1993). Delayed reproductive age, typically associated with long lived species, means that results from population management plans will not become apparent for

several years after which time corrections to management plans may not be possible. This implies that management plans need to be established based on the most information possible. Thorough knowledge of reproductive information, including seasonality in behavior and physiology, translates into more successful captive breeding. In wild populations, knowledge of reproductive biology provides field biologists important clues to the reproductive potential of individuals and growth potential of populations (Congdon et al. 1993). This same concept can translate into a captive population's situation.

In a captive environment, knowledge of reproductive hormones and the timing of reproduction for a species can allow for human intervention and manipulation at the most appropriate time. In some species, individuals can be identified as gravid by measuring hormones alone. Animals held in captivity may suffer from chronic stress, which can lead to decreased or nonexistent breeding. Injecting appropriate hormones could reverse these negative effects in certain situations. These injections may also be applicable to individuals or species that do not respond well to captive breeding situations. In some cases injected hormones can help to make animals breed (Jones 2011). Female Houston and Wyoming toads (*Anaxyrus houstonensis* and *Anaxyrus baxteri*) injected with hormones can be forced to ovulate eggs, which are then externally fertilized by males (Browne et al. 2006). In this situation concentrations and combinations of injected hormones must be calculated based on previous attempts, or knowledge of these species reproductive hormone levels. In addition, these injections must be given when the female is ready to deposit eggs. In this example, the female is completing the preparation of eggs, she is just not receiving the cue to lay them.

Giving hormone injections of oxytocin can induce individuals known to be gravid, to deposit eggs, when the female does not seem to want to lay her eggs. These injections must be

given at the correct time in order to be effective. Free ranging individuals captured while actively looking for nesting locations respond to oxytocin better than individuals captured away from nesting locations (ex. yellow belly sliders, Moss 2010). Oxytocin injections seem to be most effective in chelonian species (Ewert and Lwgieler 1978, Feldman 2007)

CHAPTER 2

INTRODUCTION

Study of long lived reptiles

Several reptile species have long-life spans and reach sexual maturity late. These life-history features may cause better quality young, greater fecundity and decreased risk of mortality as an adult (Congdon et al., 1993). Reproductive behavior, anatomy and physiology have been well studied in certain reptilian groups, such as sea turtles (in *Lepidochelys kempii*, Rostal, 2005; in *Dermochelys coriacea*, Rostal et al., 2001; in *Chelonia mydas* Jessop et al., 1999; in *Caretta caretta*, Wibbels et al., 1990, Whittier et al., 1997), while other groups are represented by a single species or genus. The American alligator (*Alligator mississippiensis*) has been well studied using histological, hormonal, and ultrasound methods for crocodylians (Lance 1989, Lance et al. 2009), garter snakes (*Thamnophis sirtalis*) have been well studied for snakes (Taylor and DeNardo 2011), and *Anolis* and *Sceloporus* have been well studied for lizards (Lovern 2011). Lizards, in particular, have been considerably understudied as a group. Of the over 5000 lizard species, detailed knowledge of reproductive patterns is only available for a small percentage (Lovern 2011).

Seasonal reproduction occurs where there is a distinct time of year for reproduction and is widespread in lizard species (Fitch 1970, Licht 1984, Pianka and Vitt 2003). Maintaining reproductive structures is energetically costly, therefore not all tissues can be maintained all year or can afford to be maintained due to environmental changes impacting the available resources. This leads to seasonal reproduction (Lovern 2011). Seasonality of reproduction is important because tradeoffs exist such that energy that is allocated to reproduction is not available for other activities such as growth or maintenance (Stearns 1989).

Reproduction in lizards is costly (Shine 1980, Whittier and Crews 1987, Crews 1998) but the nature of costs varies greatly by species and sex (Lovern 2011). Females, in general, expend more energy than males on reproduction (Orrell et al. 2004). Because reproduction is costly, it typically occurs when the cost to the adults is minimized and when the chance of survival of the offspring is maximized (Lovern 2011). Several reproductive strategies can be observed.

Multiennial reproduction occurs where the species breeds seasonally, but each animal does not breed each year. In these situations it takes longer than a year to complete egg production (ex. Sea turtles and Tuatara). This can even vary within the same species, with some populations in more favorable conditions reproducing each year, and populations in harsher environments taking more than one year per reproductive event. Another reproductive strategy is where there is no seasonality seen in the population, but on an individual basis. Each individual does not breed year round, but at any point during the year a reproductive individual can be found.

Conservation of the Guatemalan beaded lizard

The Guatemalan beaded lizard, *Heloderma charlesbogerti*, (Figure 1 & 2) is one of the rarest lizards in the world. It was first described in 1987 and immediately considered “rare” (Campbell and Vannini, 1988) and has since been found to possibly have less than 200 individuals in the wild (B. Lock per comm.). It inhabits two valleys, Rio Lagartero and Rio Motagua, located 230 km from the next closest related population of beaded lizards, *Heloderma alverezi* (Anzveto and Cambell 2010) (Figure 3). Due to its restricted range and limited population size, Zoo Atlanta’s Herpetology department has begun efforts to breed this species in captivity and produce an assurance colony. An assurance colony is not a conservation strategy, but if no animals are left by the time a conservation strategy has been established, there is no chance of successful preservation of the species. Having an assurance colony will allow for the

implementation of a reintroduction program if new restored habitat becomes available in the future. Zoo Atlanta, along with organizations in Guatemala, has secured some of the last remaining prime habitat and has been conducting field studies since 2002 (Lock 2009). This habitat is known to have beaded lizards. Other parcels of land are being restored in attempts to establish corridors between known pieces of quality habitat.

Beaded lizards and Gila monsters, both in the Helodermatidae family, are the only two types of venomous lizards in the world. Two subspecies of Gila monsters- *Heloderma suspectum suspectum* (Cope 1869) and *H. s. cinctum* (Bogart and Mattin del Campo 1956) and four subspecies of beaded lizards (Figure 3)- *Heloderma horridum horridum* (Wiegmann 1829), *H. h. exasperatum* (Bogart and Mattin del Campo 1956), *H. h. alverezi* (Bogart and Mattin del Campo 1956), and *H. h. charlesbogerti* (Cambell and Vannini 1988)(Figures 1 & 2) have been scientifically described. Recently, each of the four subspecies of beaded lizards has been elevated to species based on morphology, biogeography and molecular analysis (Reiserer et al. 2013).

Gila monsters live in the desert regions of the Southwestern United States and beaded lizard species inhabit tropical dry forests of western Mexico and Guatemala. The tropical dry forest (Figure 5) is one of the most decimated major forest types in the world, less than 2% remains and only 0.09% is afforded some type of protection (Janzen 1988). Primary threats to tropical dry forest are clearing for agriculture and cattle grazing as well as habitat fragmentation (Janzen 1988, Beck 2005, Reiserer et al. 2013). Large portions of the habitat in Guatemala have been converted to irrigated farm land and Guatemalan beaded lizards have been extirpated from more than half of their original range (Beck 2005). *Heloderma charlesbogerti* is protected by national legislation in the country of Guatemala and are listed as CITES I (the Convention on

International Trade in Endangered Species of Wild Fauna and Flora). All other beaded lizard species are listed as CITES II (Ariano-Sánchez et. al 2014). The International Union for the Conservation of Nature (IUCN) still considers all beaded lizards as a single species of least concern, however Reiserer et al.'s (2013) recent reclassification means reassessment will be needed, most likely resulting in the *H. charlesbogerti* species being listed in a threatened category (Ariano-Sánchez et. al 2014).

Little is known about reproduction in this genus, although some has been learned from captive individuals housed since the 1970's. Scientists have been reluctant to study wild populations because reproductive studies traditionally meant sacrificing the animal to histologically assess the gonads. In order to avoid sacrificing large numbers of individuals, road killed specimens and individuals collected for museum were utilized to assess reproductive condition at different times of the year (Beck 2005). In Gila monsters (*Heloderma suspectum*) spermatogenesis occurs in May and June, enlarged follicles appear from March until June, and eggs in June and August. Egg laying coincides with the beginning of summer rain in the Sonoran and Chihuahua deserts (Beck 2005). In addition, no oviductal females showed signs of additional follicular growth, indicating that females only lay one clutch per season (Goldberg and Lowe 1997). The timing of hatching in Gila monsters is contradictory. Egg incubation in captivity has ranged from 114-166 days at temperatures ranging from 26 C to 35 C (Köhler 2005, Beck 2005). In the wild Gila monster neonates are not seen for almost a year after egg laying, suggesting an incubation time of 8-10 months. It is believed that eggs possibly hatch and the neonates then overwinter in the nest before emerging the following summer. This would explain the discrepancies between captive incubation times and wild field observations of neonates (Beck 2005).

However, beaded lizards show a considerably different reproductive pattern. Captive beaded lizards collected from Mexico indicate male-female pairings occurring in October-November and egg laying occurring in November-December (Goldberg and Lowe 1997, Alvarez del Toro 1982, Ramirez Velazquez and Guichard Romero 1989). Spermatogenesis in wild beaded lizards begins in late August and continues through at least October, as determined from road kill specimens found in Mexico (Goldberg and Beck 2001). Based on a single field observation, spermatogenesis coincides with male-male competition (Beck 2005, Beck and Ramirez-Bautista 1991). Male-male competition is present in both Gila monsters and beaded lizards, and coincides with courtship, mating and spermatogenesis (Beck 2005, Ramirez-Velazques and Guichard-Romero 1989, Beck and Ramirez-Bautista 1991, Goldberg and Lowe 1997, Goldberg and Beck 2001). It is well documented that male-male combat is ritualized for both species although different behaviors are reported for each species (Beck 2005, Beck and Ramirez-Bautista 1991)(Figure 4). While some reproductive information is available, it is extremely limited for all helodermatid species. No hormones have been analyzed, and individual animals have not been followed through a complete cycle in captivity or the field.



Figure 1- Adult Guatemalan beaded lizards in outdoor summer holding enclosure. Two of the individuals are battling in the foreground. Guatemalan beaded lizards are identifiable by the distinct yellow bands on the tail and limited amounts of yellow spots on a black background body color. Photograph by Wade Carruth.



Figure 2- Hatchling Guatemalan beaded lizard (ID-12R009) from Zoo Atlanta, hatched in 2012. This is the first Guatemalan beaded lizard to hatch at Zoo Atlanta. Incubation took 187 days at 26.7 degrees Celcius (80 degrees Fahrenheit). Photograph by Wade Carruth.

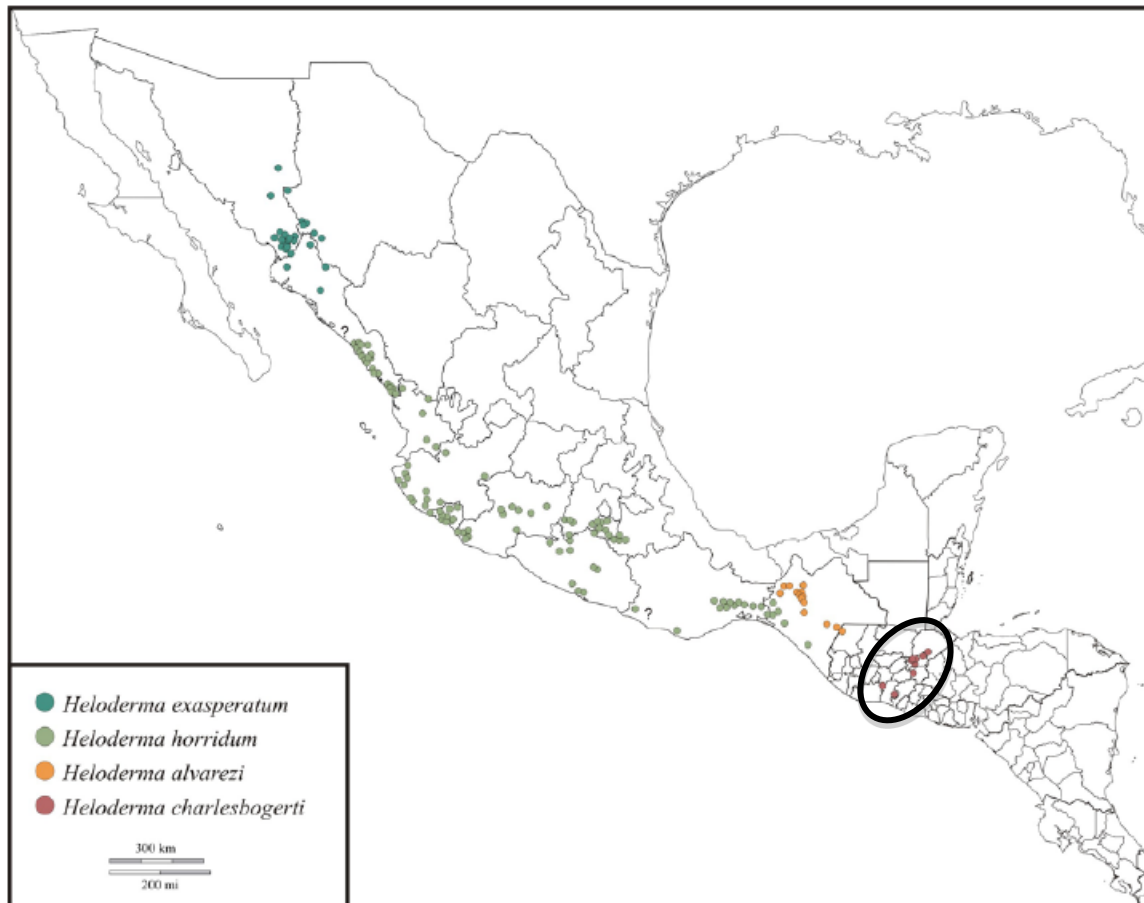


Fig. 7. The distribution of beaded lizards in Mexico and Guatemala. Colored dots represent verified sightings (populations) and museum records. Note the fragmented populations of all four species, which closely approximates the patchy distribution of seasonal dry tropical forests (see map in Brown and Lowe [1980]). See text for explanation of question marks (“?”) and other details.

Figure 3- Ranges of the 4 species of beaded lizard. *H. charlesbogerti* is located in the bottom right (circled), separated from remaining populations by 230km of non-suitable habitat. Ranges closely follow the distribution of tropical dry forests. (Reiserer et al. 2013)



Figure 4- Two male Guatemalan beaded lizards engaging in ritualized battle. Males arch themselves with their head and tail touching the ground while trying to push the other male to the ground by forming a taller arch. Battles can last anywhere from a few minutes or continue for extended periods of 15-16 hours (Beck 2005). These fights rarely result in severe injuries, but can result in small wounds from bites and damage to tail tips from rough terrain. Photograph by Brad Lock.



Figure 5- Photograph of tropical dry forest in eastern Guatemala during the wet season.

Photograph by Brad Lock.

Objectives

The purposes of this study were to:

1. Delineate the reproductive hormone cycle of *H. charlesbogerti* by monitoring seasonal steroid and calcium cycles, vitellogenesis, ovarian follicular growth, egg production and nesting activities.
2. Correlate testosterone, estradiol, corticosterone and calcium levels with reproductive condition determined by ultrasonography.
3. Increase captive reproduction of the Guatemalan beaded lizard, for other institutions to have exhibit animals, thereby increasing awareness and education about the species, and also for possible reintroductions to Guatemala.

METHODS

The Guatemalan beaded lizards housed at Zoo Atlanta provide an excellent opportunity to study a rare and unique lizard species. These animals, five at the beginning of the study, 14 at the end of the study, are the only Guatemalan beaded lizards legally outside of Guatemala (Table 1). Sampling began in March 2011 with five individuals, and in September of 2011 nine animals were transferred from the San Diego Zoo to Zoo Atlanta. After limited reproductive success and increased interests by the Zoo Atlanta team in this species, these additional animals were acquired in hopes of increasing reproductive success.

Animals and housing

Animals were housed at Zoo Atlanta's Herpetology department. Initially three males and two females (one captive bred) were available for blood sampling and ultrasound exams. After seven months of sampling, nine additional animals (six males and three females) were transferred from the San Diego Zoo and added to the study (total N=14). The captive population

was comprised of nine wild caught founder individuals (six males and three females) and five captive bred offspring (three males and two females, produced at San Diego). Founder males averaged 1.55kg and founder females averaged 1.69kg at the beginning of the study (March 2011). Two captive reproductive events occurred at the San Diego Zoo resulting in three hatchlings in 2003 and two in 2006 (Johnson 2011). Captive bred offspring from 2003 (two males and one female) had an average mass of 1.37kg and offspring from 2006 (one male and one female) had an average mass of 0.57kg at the beginning of the study (Table 1).

At Zoo Atlanta, a breeding group of ten individuals (nine founders and 2003 hatch female) were housed in a single large 24ft (W) x 12ft (D) x 10ft(H) enclosure (Figure 6) with double layer 1x1 inch hardware cloth and double door entry during summer months in order to maintain UV exposure and temperature requirements. Animals were outside for approximately six months of the year, when nighttime temperatures remained above 10° Celsius (50° Fahrenheit) (Figure 7). This group was comprised of six males (all founders) and four females (three founders and one captive bred from 2003). Being housed together allowed for natural male-male competition and mate selection. Animals were closely monitored and male-male combat is a ritualized battle that rarely results in physical injuries (Figure 4, Beck and Ramirez-Bautista 1991). The remaining four animals were all captive bred (three males and one female) and were housed separately (Figure 6). During winter months animals were housed indoors in large 4ft (W)x4ft (D)x2.5ft (H) modified plastic shipping containers with custom wood/hardware cloth tops (Figure 6) and heating elements (80° hot spot, no UVB). Winter months had 1-3 individuals housed together based on sex and size. Gravid females were housed individually to allow for egg laying. Females that were not gravid were housed with an individual male, while other males were housed in groups.

Lizards were fed a varied diet of rodents, day old chickens, and quail eggs. During summer (May-October) months animals were fed 2-3 food items once per week, feedings were reduced to once every two-three weeks during fall (November-February). Animals were then not fed for one-two months, March-April (dry season in Guatemala), in order to simulate natural conditions. Body condition and reproductive status are also considered when feeding. Females that laid eggs were generally fed larger meals, in order to regain body condition after oviposition. Care was taken not to over feed animals as over weight individuals can be unhealthy. The hips should be slightly visible on a healthy individual; individuals seen in the wild always appear thin (B. Lock, per comms.). Water availability was also modified in order to mimic what animals would encounter in the wild, with reduced water availability, during the winter months. Water is removed for three days a week during what would be the dry season in Guatemala (Beck 2005).

All eggs were incubated at 26.7 degrees Celcius (80 degrees Fahrenheit). The one successful incubation took 187 days. Eggs were incubated while suspended above saturated substrate, so that no moisture was in direct contact with the eggs (SIM- Suspension Incubation Method, Squamata Concepts, New York, New York). Eggs were in 100% relative humidity.

Table 1- List of individuals housed in Zoo Atlanta's collection during the study with information on sex, where the animals originated, year of capture or hatching, number of sampling events, and body mass at the beginning of the study.

ID	Sex	Source	Captured or Hatch Year	Number of Sampling Events	Weight in Kg (at entrance of study)
A06100	Female	Wild	1992	6	1.709
A06103	Male	Wild	1992	6	1.324
A06104	Male	Wild	1992	13	2.16
A06105	Female	Wild	1997	13	1.81
A06106	Male	Wild	1997	6	2.047
A06107	Male	Wild	1997	6	1.283
A06108	Male	Wild	1984	13	1.76
A06109	Male	Wild	1997	13	2.3
A06110	Female	Wild	1997	6	1.557
A96101	Female	Captive Bred	2003	13	1.7
11R059	Male	Captive Bred	2003	6	1.234
11R060	Male	Captive Bred	2003	6	1.185
11R061	Male	Captive Bred	2006	6	0.625
11R062	Female	Captive Bred	2006	6	0.514



Winter holding
containers

Figure 6- Summer holding for the breeding group of Guatemalan beaded lizards (24ftx12ftx10ft). Walls are made of two layers of 1 inch by 1 inch welded hardware cloth. Entry points consist of two doors in order to provide double containment. Other individuals are kept in separate enclosures pictured in foreground (three white shipping containers, labeled). These white tubs (4ftx4ftx2.5ft) with lid (1in by 1in welded hardware cloth with wooden frame), are also used indoors for winter holding with 1-3 individuals per container with addition of artificial heat (no UVB). Photograph by Wade Carruth.

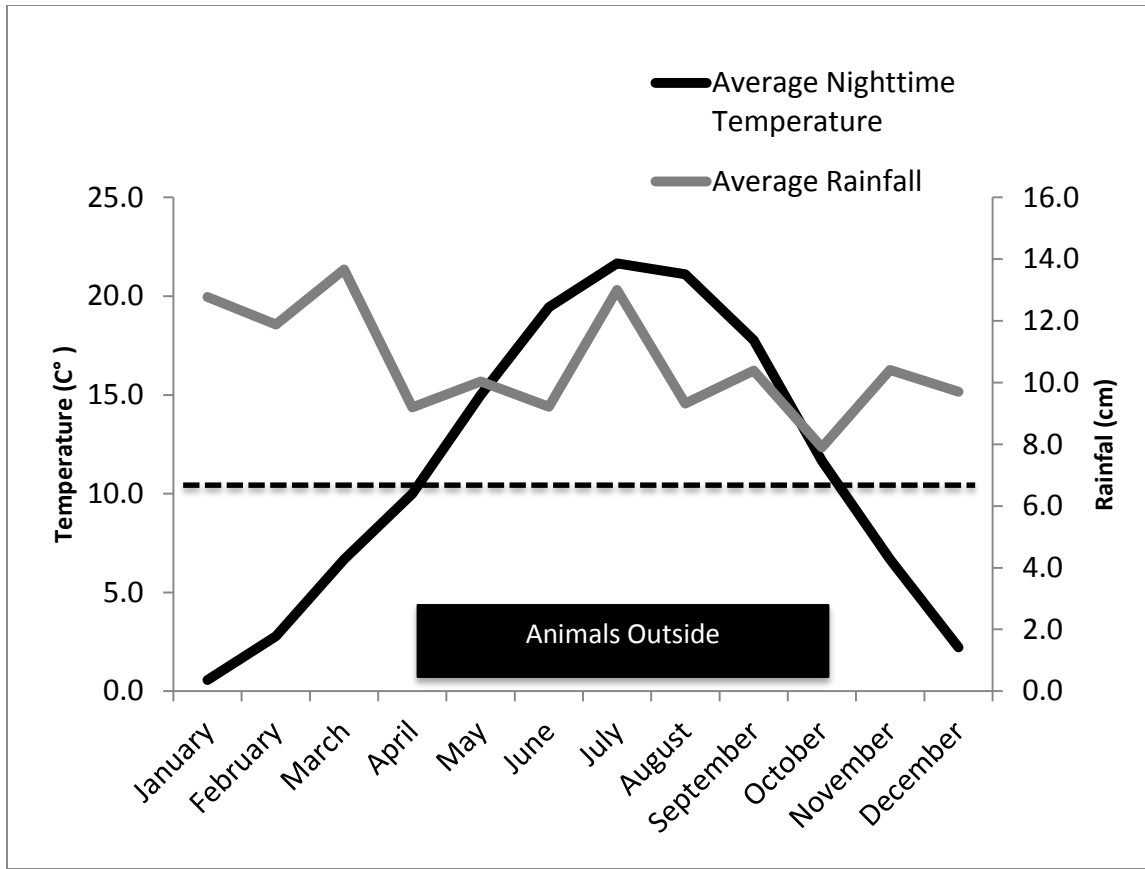


Figure 7- Average monthly temperatures for Atlanta, Georgia, animals are left outside (day and night) during all months where temperatures remain above 10 degrees Celcius (50 degrees Fahrenheit, indicated by black box and dashed line). Typically, lizards are outside from mid-April through mid-October, approximately 6 months. Lizards are not moved in and out on a daily basis, but only moved once minimum temperature requirements will continue to be met for the season.

Blood sampling

Blood samples were collected monthly from March 2011 through March of 2012 on the original five individuals. Sampling of the transferred animals from San Diego did not begin until September 2011 (seven month delay). Samples were collected by zoo staff immediately after handling began (< 3 mins), in order to minimize the stress response (Elsley et al., 1991, Romero and Reed 2005). Sampling occurred from 0900-1100. Approximately two mL of blood was taken from the ventral caudal vein in the base of the tail (4-6 cm from cloaca) using a 3 mL syringe with 21 gauge 1.5 inch needle while an assisting person was restraining the animal at approximately 45° to horizontal (Figure 8). Following blood collection, samples were transferred to 4 mL sodium heparin vacutainers and kept on ice until all 14 individuals had been sampled. Samples were centrifuged at 5000 rpm for 10 minutes. Plasma was stored separately from red blood cells frozen at -20° Celsius until analysis.



Figure 8- Top- Dr. Brad Lock (left) and Keeper III Luke Wyrwich (right) performing blood draw on a Guatemalan beaded lizard with a 3 mL syringe and 21 gauge 1.5 inch needle. An average of 2mL was taken each month. Bottom- close up of ventrel caudel vein venipuncture. Photographs by Wade Carruth.

Ultrasound imaging and X-ray

A Sonosite portable ultrasound (Vet 180 plus) unit with a curvilinear probe (C11/7-4 MHz Transducer) was utilized to non-invasively observe the reproductive structures on females (Rostal et al. 1990, Robeck et al. 1990) (Figure 9). This technique has been used on a wide range of species since the 1970s (Gilman and Wolf 2007). Visible structures were measured using the ultrasound machine's internal calipers. Images were saved for later review (see Figures 20-25). We were not able to observe testis.

Radiographs of gravid females were taken by zoo staff using an Eklin Mark V Digital Radiography System with settings of 65 kVp and 2 mAs (Figure 10).



Figure 9- Dr. David Rostal with ultrasound transducer imaging a female Guatemalan beaded lizard. Animal is held by Dr. Brad Lock. Follicular development is observed from ventral surface, just cranial to hind limbs. Photograph by John Parrish.

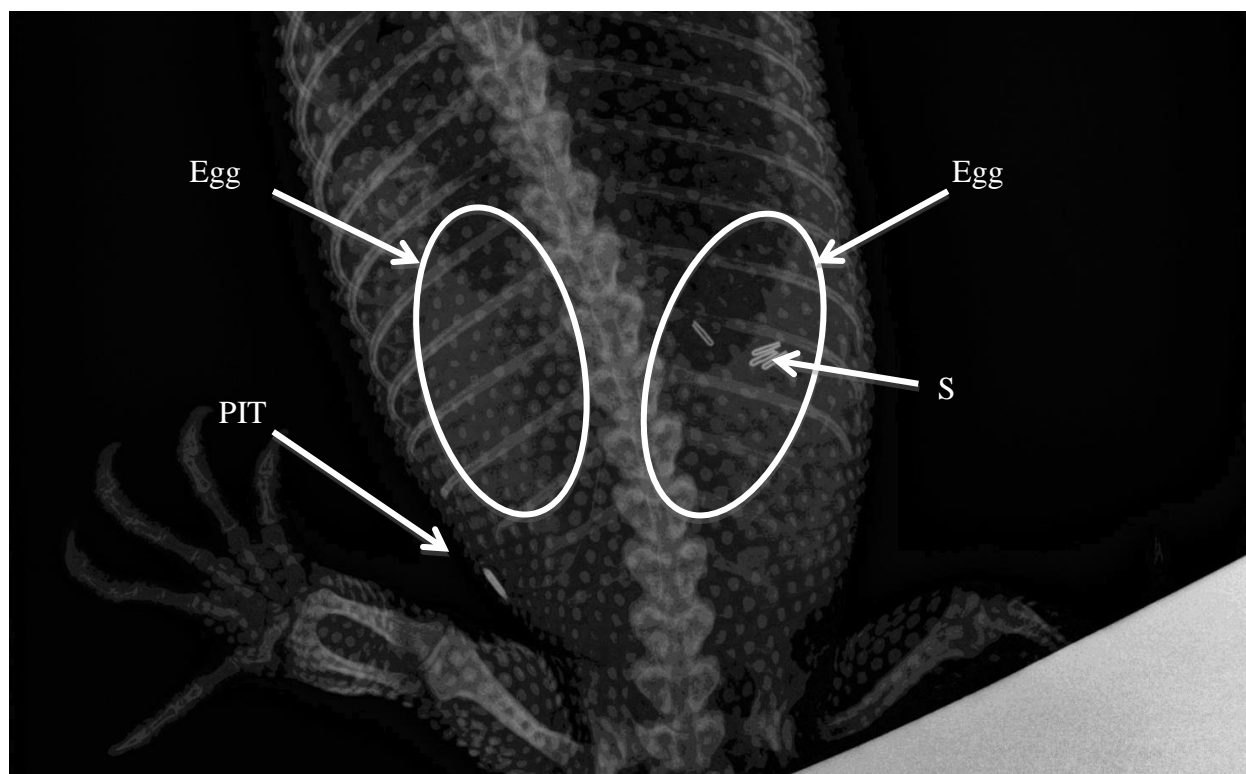
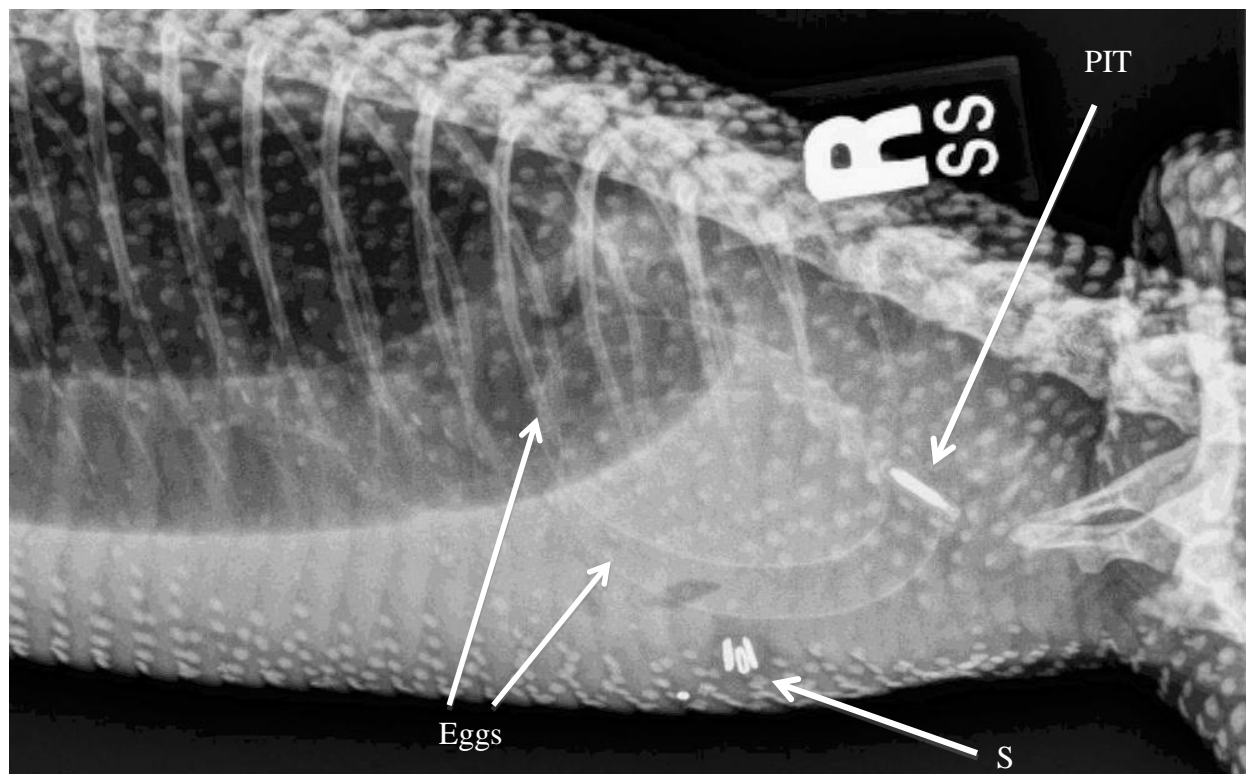


Figure 10- Lateral (top) and dorsoventral (bottom) radiograph of female Guatemalan beaded lizard A06105 at Zoo Atlanta, taken September 27, 2011. Osteoderms are visible as small white opaque structures on entire body. Two eggs are faintly visible (outlined in white) in the abdomen cranial to hind limbs and posterior to lungs. Staples on right side of the image (S) are from a partial ovariectomy performed several years prior. A Passive Integrative Transponder (PIT) microchip is also visible on left side of image. Radiographs were taken using an Eklin Mark V Digital Radiography System (65 kVp and 2 mAs).

Hormone and Calcium Analysis

Plasma testosterone (Catalogue Number 582701), estradiol (Cat. No. 582251) and corticosterone (Cat. No. 500655) were measured via enzyme linked immnosorbant assays (ELISA) purchased from Cayman Chemical Company (Cayman Chemical © Ann Arbor, MI), and performed at Georgia Southern University.

Dilutions of samples varied with time of year, individual, and assay being run. Dilutions were chosen so that the percentage of bound analyte fell within the most sensitive portion of the standard curves (20-80% B/B₀). Dilutions for testosterone in males were 1:10 (15µL of plasma), 1:20 (10µL of plasma), 1:40 (10µL of plasma) and 1:80 (10µL of plasma). Dilutions for estradiol in females were 1:0.5 (230µL of plasma) and 1:1 (115µL of plasma). Dilutions for Corticosterone in both sexes were 1:8 (15µL of plasma) and 1:20 (10µL of plasma). Assays were completed using plasma samples with a simple ether extraction for purification.

Approximately 1mL of ether was added via repeat dispenser to a predetermined volume of plasma in a clean disposable culture tube and vortexed for 30 sec, allowing the phases to separate. Then the sample was snap frozen, using dry ice. The steroid molecules become suspended in the ether, which has a much lower freezing temperature, while the plasma freezes.

The ether was then poured into a new clean disposable culture tube, leaving the plasma in the original tube, thereby giving a more purified sample. Samples were allowed to dry overnight under a fume hood. The dried samples, now residue on the walls of the vial, are then used to perform the assay according to manufacturer's directions. The samples were reconstituted with the provided EIA buffer, and added to an antibody coated 96 well microplate, followed by the reconstituted acetylcholinesterase conjugate (tracer) and antiserum, which were shaken and incubated at room temperature for one or two hours (depending on type of assay). During incubation, competition between the tracer and the hormone molecules in the sample over a limited number of antiserum molecules occurs. After either a tracer molecule or a molecule from the sample binds to the antiserum, the entire complex then binds to the wall of the plate. Because the concentration of tracer remains constant while the concentration of hormone in the sample varies, the amount of tracer that binds to the antiserum will be inversely proportional to the amount of hormone in the sample. Post-incubation, the plate was washed and the bound enzyme conjugate (tracer) will be detected through the addition of reconstituted Ellman's reagent, generating an analyzable color after 30-60 minutes. Quantitative results were obtained by the measurement and comparison of absorbance readings between the samples and known standards with an automated microplate reader model EL311S (BioTek Instruments Inc., Winooski, Vermont). The reader provided an absorbance report consisting of the microplate's optical density values, corrected by single or dual wavelength, blanking and factoring. The extent of color development is inversely proportional to the amount of hormone in the sample (Figure 11). After completion of the assay, absorbance readings were entered into a Microsoft excel program (provided by Cayman Chemical Company) and concentrations were automatically calculated.

A standard curve is generated for each assay based on the known concentrations of hormone in the assay's serial dilution. Hormone concentrations in each plasma sample are calculated from the linear regression equation of the standard curve. Hormone assays were validated for the study species by running a pooled sample serially at three or more dilutions in the same assay, then comparing regressions of the kit's standard curve with the serial dilution of pooled sample (Figure 12). Parallel regression lines indicate no interfering reagents. Estradiol was not easily measured, most likely due to the low amounts of circulating hormone. Due to the large number of samples that were run, multiple assays were run for each hormone analyzed. Inter-assay coefficients of variation were 17.2% for testosterone, 12.4% for corticosterone and 21.1% for estradiol. Intra-assay variation was calculated to be less than 20% coefficient of variation for all samples. Individuals that were higher than 20% coefficient of variation were measured again in subsequent assays until a CV of <20% was achieved.

Measurements are most accurate when the percentage of bound conjugate is 50%. As samples get above 80% bound and below 20% bound measurements become inaccurate, and are not used. These samples were re-analyzed at a different dilution that falls between 20 and 80% bound.

The testosterone (Cat. No. 582701, Cayman Chemical © Ann Arbor, MI) antibody had cross reactivity with 19-Nortestosterone of 140%, 5 α -dihydrotestosterone of 27.4%, 5 β -dihydrotestosterone of 18.9%, Methyl Testosterone of 4.7%, Androstenedione of 3.7%, 11-keto Testosterone of 2.2%, 5-Androstenediol of 0.51%, *Epi*-Testosterone of 0.2%, Progesterone of 0.14%, Testosterone Enanthate of 0.11%, Androsterone of 0.05%, Androsterone Sulfate of 0.04%, Testosterone Sulfate of 0.03%, DHEA Sulfate of 0.02%, Estradiol of <0.01%, and Testosterone Glucuronide of <0.01%. The estradiol (Cat. No. 582251, Cayman Chemical © Ann

Ann Arbor, MI) antibody had cross reactivity with Estradiol-3-sulfate of 14.5%, Estradiol-3-glucuronide of 14%, Estrone of 12%, Estradiol-17-glucuronide of 10%, Estriol of 0.30%, 5 α -dihydro Testosterone 0.06%, Ethynyl Estradiol of 0.05%, 5-Androstan-17 β -ol-3-one of 0.02%, Androstenediol of 0.02%, Aldosterone of <0.01%, Cortisol of <0.01%, 17 α -Estradiol of <0.01%, Estradiol-3-benzoate of <0.01%, Estradiol-17-glucuronide of <0.01%, Estradiol-17-sulfate of <0.01%, Hydrocortisone of <0.01%, Maturation-Inducing Steroid (salmonid) of <0.01%, Progesterone of <0.01%, 17 α -hydroxy Progesterone of <0.01%, Testosterone of <0.01%, DHEA of <0.01%, and DHEA Sulfate of 0.0024%. The corticosterone (Cat. No. 500655, Cayman Chemical © Ann Arbor, MI) antibody had cross reactivity with 11-Dehydrocorticosterone of 11%, 11-Deoxycorticosterone of 7%, Progesterone of 0.31%, Cortisol of 0.17%, Aldosterone of 0.06%, Testosterone of 0.03%, Prenenolone of 0.02%, 5 α -DHT of 0.01%, Androstenedione of <0.01%, Cortisone of <0.01%, DHEA of <0.01%, and DHEA-S of <0.01%.

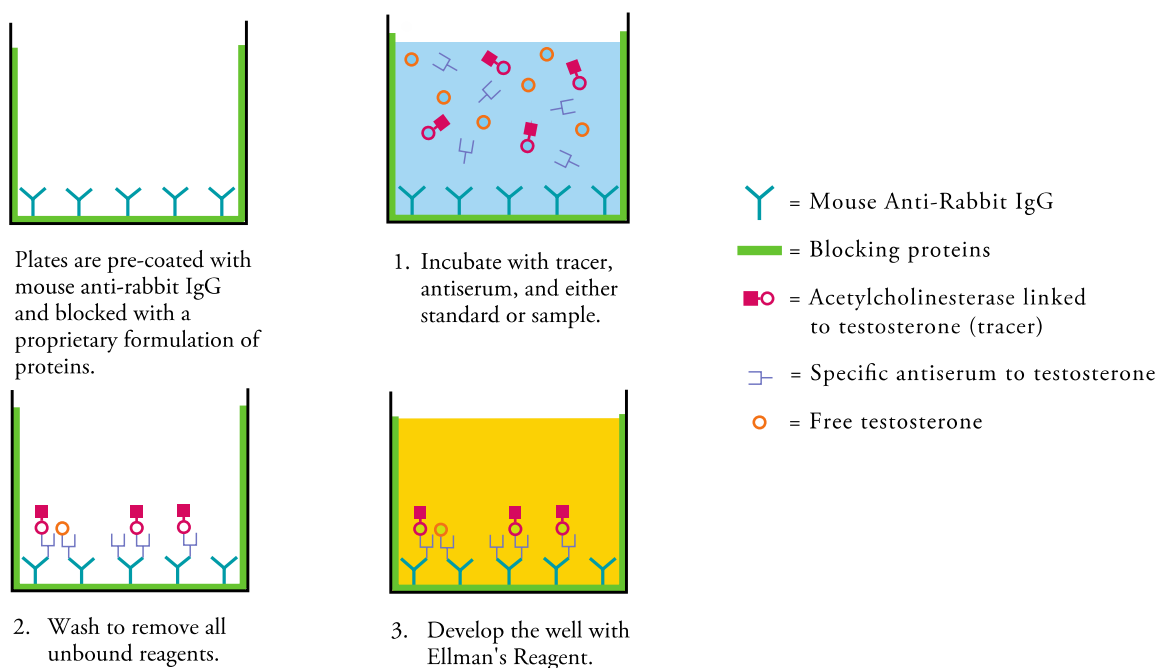


Figure 11- Schematic of EIA kit, from Cayman Chemical, Ann Arbor, MI. Plates are coated with binding sites. Tracer, antiserum, and extracted sample are added to the wells. Hormone molecules in the sample compete with a known concentration of tracer for a limited number of antiserum molecules. After an antiserum molecule is bound, it then binds to the binding sites on the wall of the plate. The plate can then be developed to reveal the ratio of sample hormone molecules to tracer molecules. Ellman's reagent is used to develop the plate, turning each well a shade of yellow. Because the number of tracer molecules remains constant, the concentration of hormone in the sample is inversely proportional to the color absorbance reading.

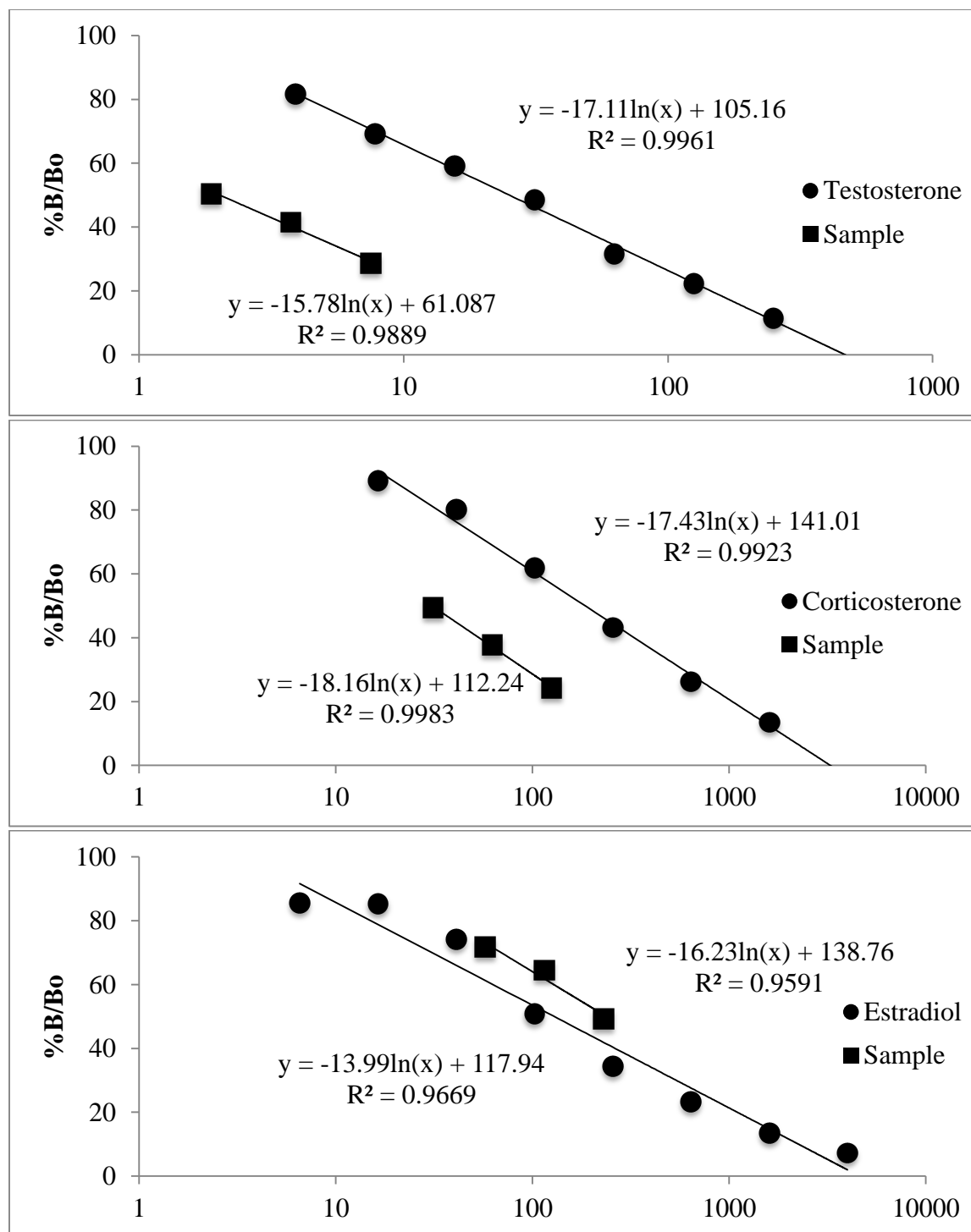


Figure 12- Displacement curves between standard and plasma dilution series for testosterone (top), corticosterone (middle), and estradiol (bottom). Note that lines are parallel for testosterone, corticosterone, and estradiol.

Plasma calcium was measured as an indicator of vitellogenesis by dry slide technology utilizing an IDEXX VetTest Chemistry Analyzer.

Seasonal variation in circulating plasma steroid levels was determined using repeated measures analysis of variance.

RESULTS

The breeding group of Guatemalan beaded lizards displayed a distinct seasonal cycle with mating during August-September and egg laying in October-December. Male-male combat was observed on numerous occasions throughout the study, including the non-breeding season, especially after introducing lizards to a new environment. Seasonal changes in steroid levels were observed in both males and females.

Testosterone levels in male Guatemalan beaded lizards varied significantly across months (dF: 8,12; F: 5.5127; $p < 0.0001$), with peak levels during July and August (Figure 13). Male testosterone levels ranged from 369.9 ± 80.3 pg/mL during nesting season (December) to 7644.8 ± 1057.9 pg/mL just prior to breeding (August). Three male individuals were available for sampling from March 2011 until August 2011 when six additional individuals were added for the remainder of the study (until March 2012).

Female estradiol did not show significant differences among months; however ovulatory individuals showed spikes (≥ 100 pg/mL) in August and December (Figure 14 & 15). Standard errors are high during the months of August and December, months where individuals had ovulatory spikes while other individuals had minimal levels (Figure 14). Looking at the four adult females individually allows for visualization of the different timing of ovulation (Figure 15). Two female individuals were available for sampling from March 2011 until August 2011 when two additional individuals were added for the remainder of the study (until March 2012).

Circulating calcium levels differed significantly between sexes but not between months (dF: 1,12; F: 7.1827; p= 0.0354). Females had higher levels of circulating calcium ranging from 14.8 mg/dL in March 2011 up to 20.6 mg/dL in June 2011. Calcium levels remained high through September 2011, during vitellogenesis, after which it dropped to 13.2 mg/dL. Male calcium levels remained lower and constant across the year (12.0-13.9 mg/dL). Three males and two females were available for sampling from March 2011 until August 2011 when six males and two females were added for the remainder of the study (until March 2012) (Figure 16). Similar to estradiol, looking at the four adult females allows for visualization of the difference in timing among individuals (Figure 17). Peaks in estradiol have corresponding elevated calcium levels for the same individual (Figures 15 & 17).

Corticosterone levels in males ranged from 975 pg/mL in May 2011 to 2600 pg/mL in March 2012. All months were below an average of 5000 pg/mL. Corticosterone levels in females ranged from 750 pg/mL in February 2012 to 5050 pg/mL in August 2011 and 4550 pg/mL in January 2012. Twelve of the 13 months were under an average of 5000 pg/mL (Figure 18). 5000 pg/mL is the point at which the animal begins showing signs of a stress response (Romero and Reed 2005). Individual females show increased levels of corticosterone while gravid (Figure 19), these increased levels correspond with estradiol (Figure 15) and calcium levels (Figure 17). Three males and two females were available for sampling from March 2011 until August 2011 when six males and two females were added for the remainder of the study (until March 2012).

Reproductive structures were easily visible in female individuals. Four phases of ovary development were observed:

1. Previtellogenic follicles (<1 cm), seen from March to May and again from January to March. Previtellogenic follicles are indicated by a black spherical appearance, as sound waves are passing through the follicle (anechoic).
2. Vitellogenic follicles (>1cm), present from May to December. Vitellogenic follicles are indicated by a grainy spherical appearance created by yolk platelets (vitellogenin) reflecting sound waves (echoic).
3. Eggs were observed from August to December. Eggs are indicated by a spherical echoic structure (yolk) inside of a thin echoic oval line (egg shell).
4. Atretic follicles were observed from December to March. Atretic follicles are anechoic and typically large (>2.0cm) and misshapen.

Ultrasound examinations began in early March of 2011, when previtellogenic follicles with an average diameter of 0.67cm were observed. Previtellogenic follicles continued to be seen at the end of March (0.75cm) and April (0.98cm). Beginning in May 2011 vitellogenic follicles were observed (1.11cm), and continued through June (1.60cm), early August (2.44cm). By late August both vitellogenic follicles (1.93cm) and eggs were observed (2.49cm). Both vitellogenic follicles and eggs continued to be observed in September and October. September vitellogenic follicles averaged 2.17cm and eggs averaged 2.80cm. October vitellogenic follicles averaged 2.23cm and eggs averaged 3.62cm. In the month of November all phases of ovary development were observed. Previtellogenic follicles averaged 0.49cm, vitellogenic follicles averaged 3.12cm, eggs averaged 2.77cm and atretic follicles averaged 2.59cm. By January all eggs are gone, previtellogenic follicles (0.49cm) and atretic follicles (2.40cm) were observed. In February no previtellogenic follicles were observed, most likely due to their small size at this time of year, however atretic follicles (2.40cm) were seen. In March, previtellogenic follicles (0.65cm) and a

few remaining atretic follicles (2.90cm) were imaged. Ultrasound measurements are summarized in Figure 20.

During the 2011 reproductive season, two clutches of eggs were laid by the four adult female Guatemalan beaded lizards at Zoo Atlanta. Individual A06105 laid five eggs from September 23, 2011 to October 13, 2011. In captivity eggs are rarely laid together all at once. Most reproductive events reported for this genus include single eggs being deposited on top of substrate one egg per day. Only one of five eggs hatched successfully on March 27, 2012 (Figure 2). Individual A96101, captive bred in 2003, laid five eggs from December 12, 2011 to December 25, 2011; all appeared misshapen and under calcified, none hatched. Individuals A06100 and A06110 (transferred from San Diego) did not lay eggs in 2011, however did show follicular growth.

In March 2011 multiple previtellogenic follicles (<1.0cm diameter) were seen in individual A06105. Bladder was also visible (Figure 21). In May 2011 slightly larger previtellogenic follicles (~1cm) were observed (Figure 22). In June 2011 vitellogenic follicles were observed to be >2.0cm. (Figure 23). In August 2011 an egg with a diameter of 2.5cm was imaged. Egg yolk and egg shell are both visible (Figure 24). Then, in September 2011 the eggs were more developed, indicated by a water cavity (Figure 25). After oviposition, in November 2011 small (<0.5cm) previtellogenic follicles were observed (Figure 26). These follicles will go through vitellogenesis the following reproductive season.

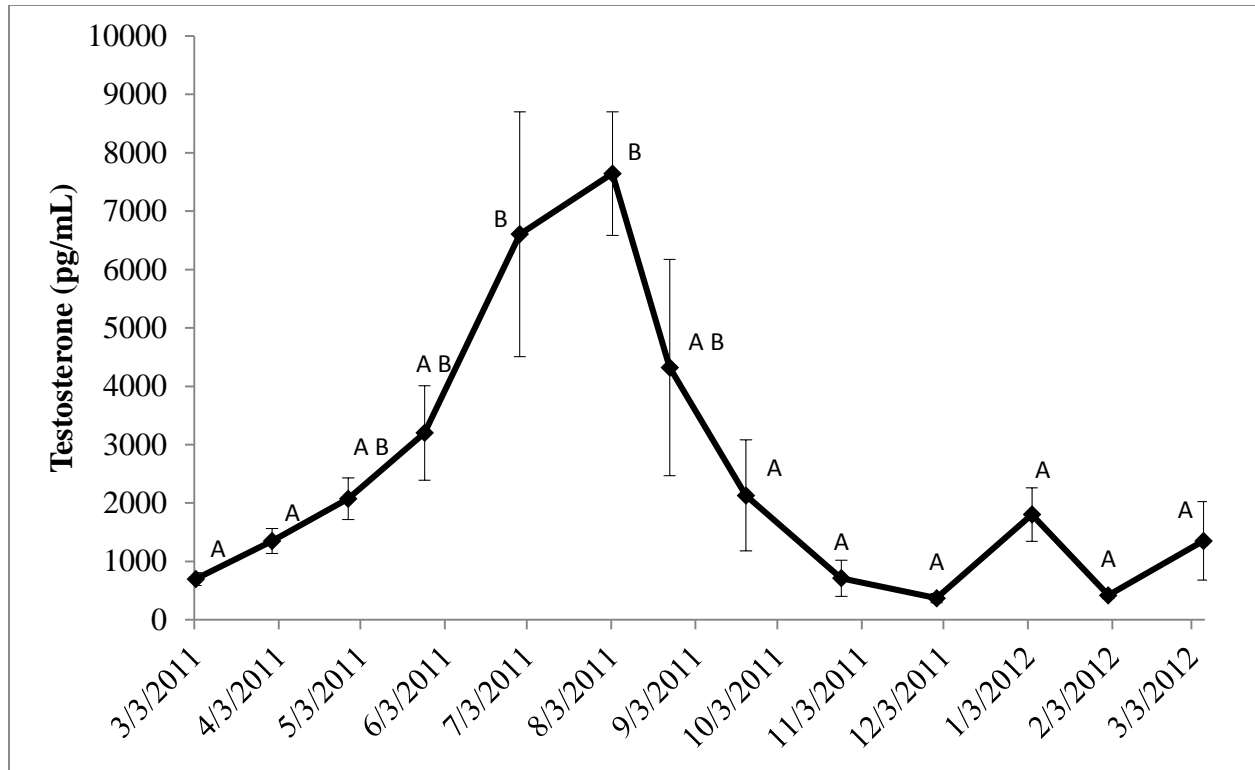


Figure 13- Mean monthly levels of circulating plasma testosterone levels (pg/mL) of male Guatemalan beaded lizards from March 2011 to March 2012. Values are means \pm standard error. Data points with different letters are significantly different. Sample size was 3 through August 2011 then expanded to 9 for remainder of the study. (dF: 8,12; F: 5.5127; $p < 0.001$)

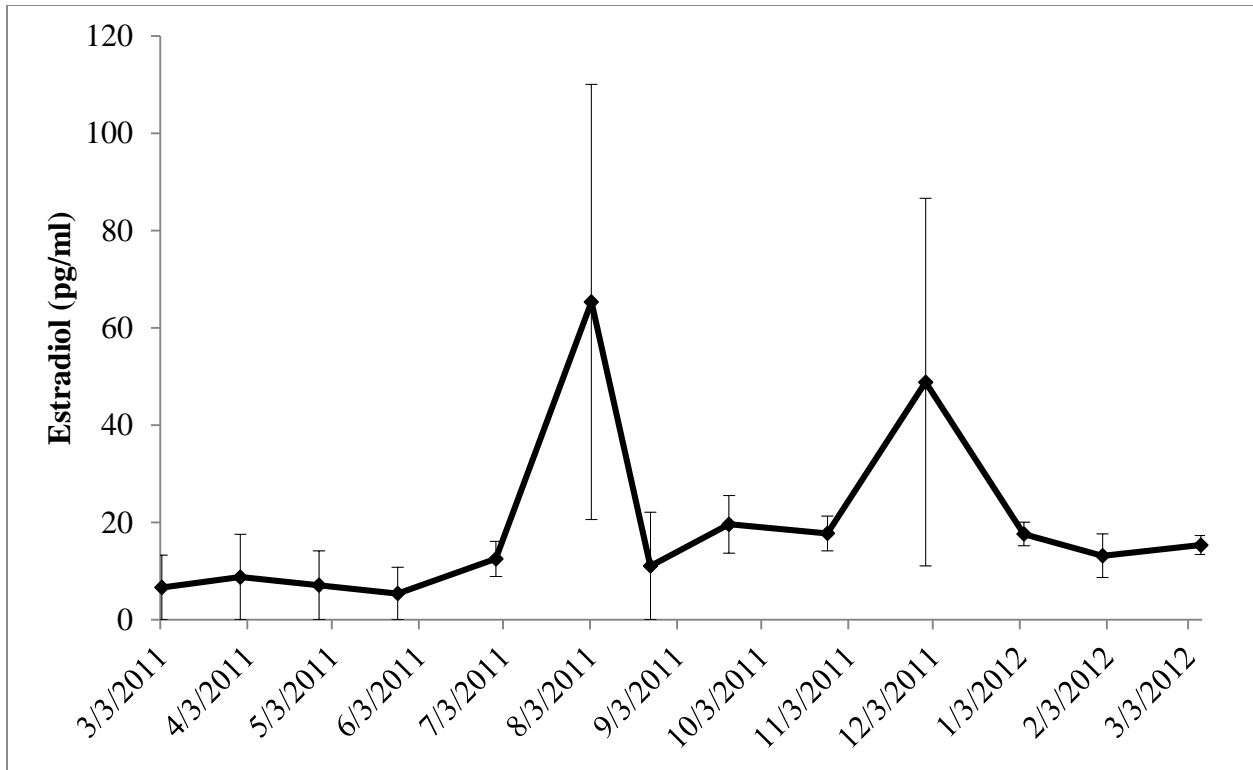


Figure 14– Mean monthly levels of circulating plasma estradiol levels (pg/mL) of female Guatemalan beaded lizards from March 2011 to March 2012. Values are means \pm standard error. Sample size was two until August 2011 then expanded to four, for the remainder of the study. Large standard errors are due to elevated levels in a single individual.

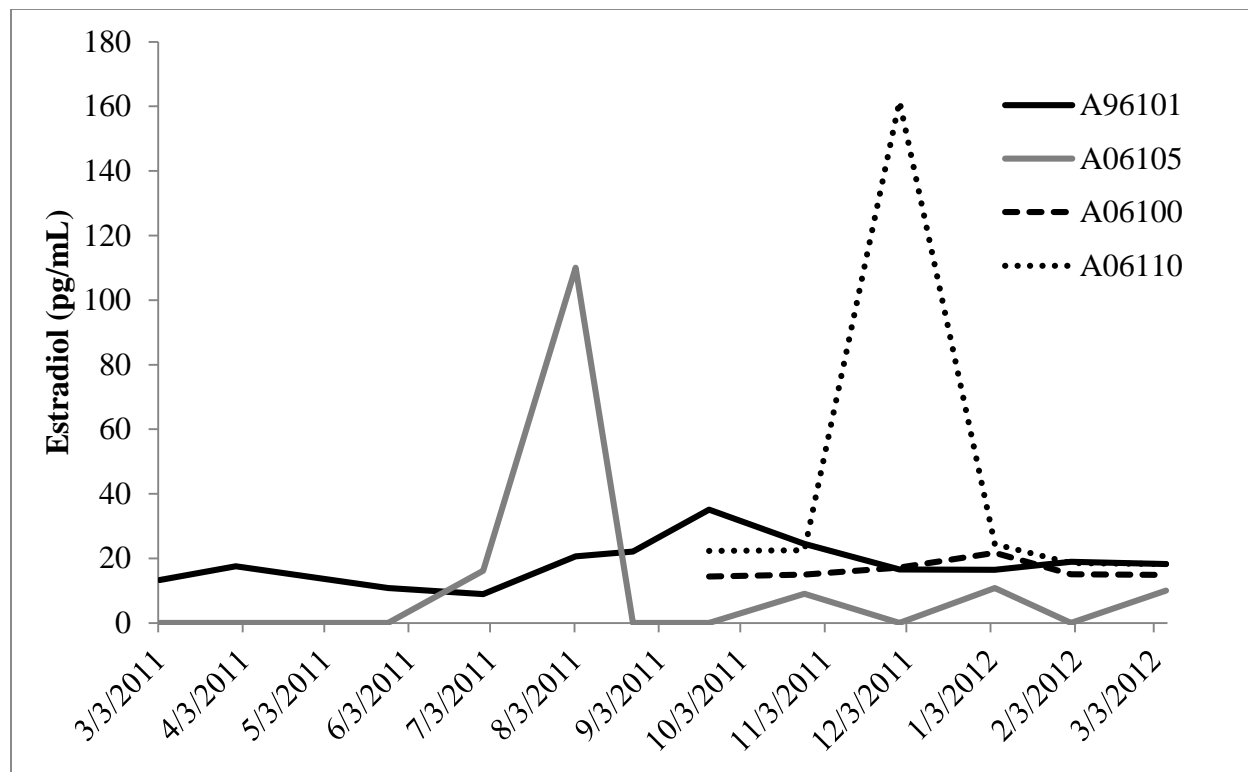


Figure 15- Individual estradiol (pg/mL) levels of the four adult female Guatemalan beaded lizards in the study from March 2011 to March 2012. Two individuals show peaks, A06105 and A06110. Individual A06105 laid five eggs from September 23, 2011 to October 13, 2011 and A96101 laid five eggs from December 12, 2011 to December 25, 2011. Zero values were entered for samples with extremely small amounts of estradiol that were undetectable. Individuals A96101 and A06105 were available for the entire study, individuals A06100 and A06110 were sampled for the first time on September 21, 2011.

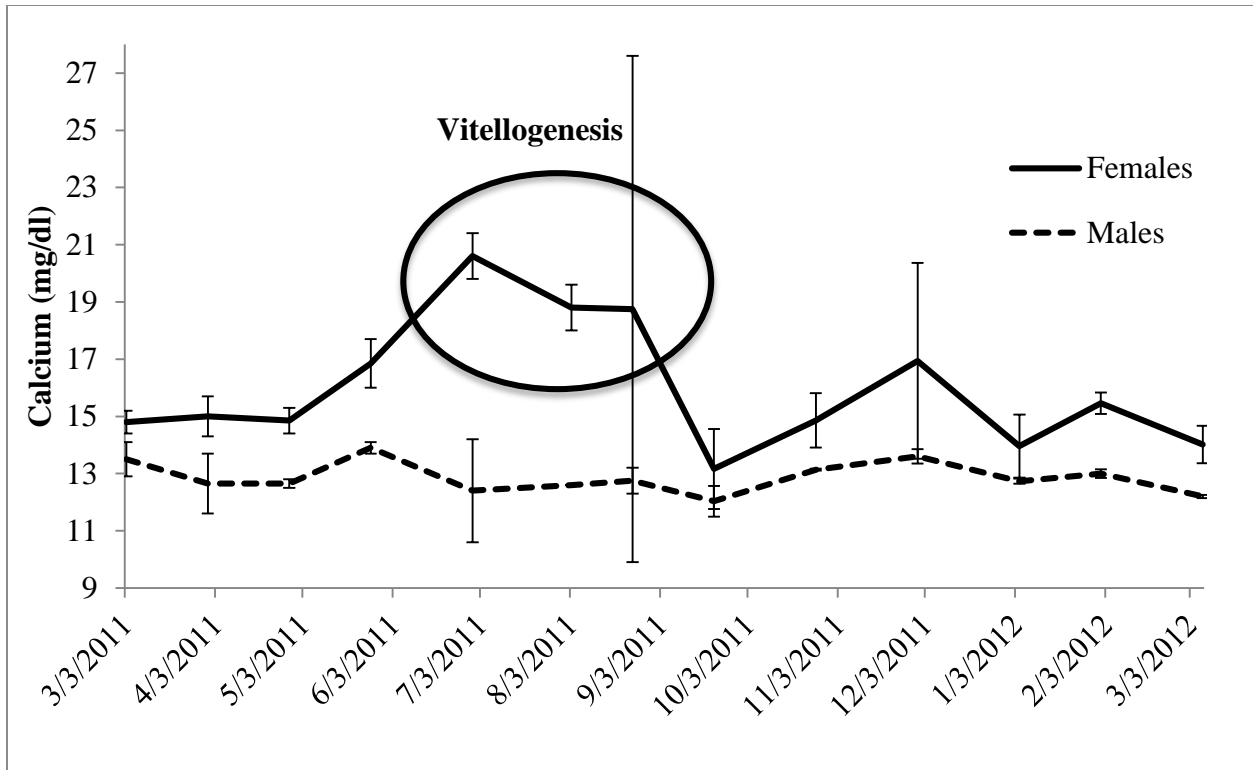


Figure 16- Mean monthly calcium levels of male and female Guatemalan beaded lizards. Values are means \pm standard error. Females had significantly higher calcium levels across months (dF: 1,12; F: 7.1827; $p=0.0354$). A pre-vitellogenic surge in mean monthly calcium levels is seen in females from July to September 2011 (circled and labeled).

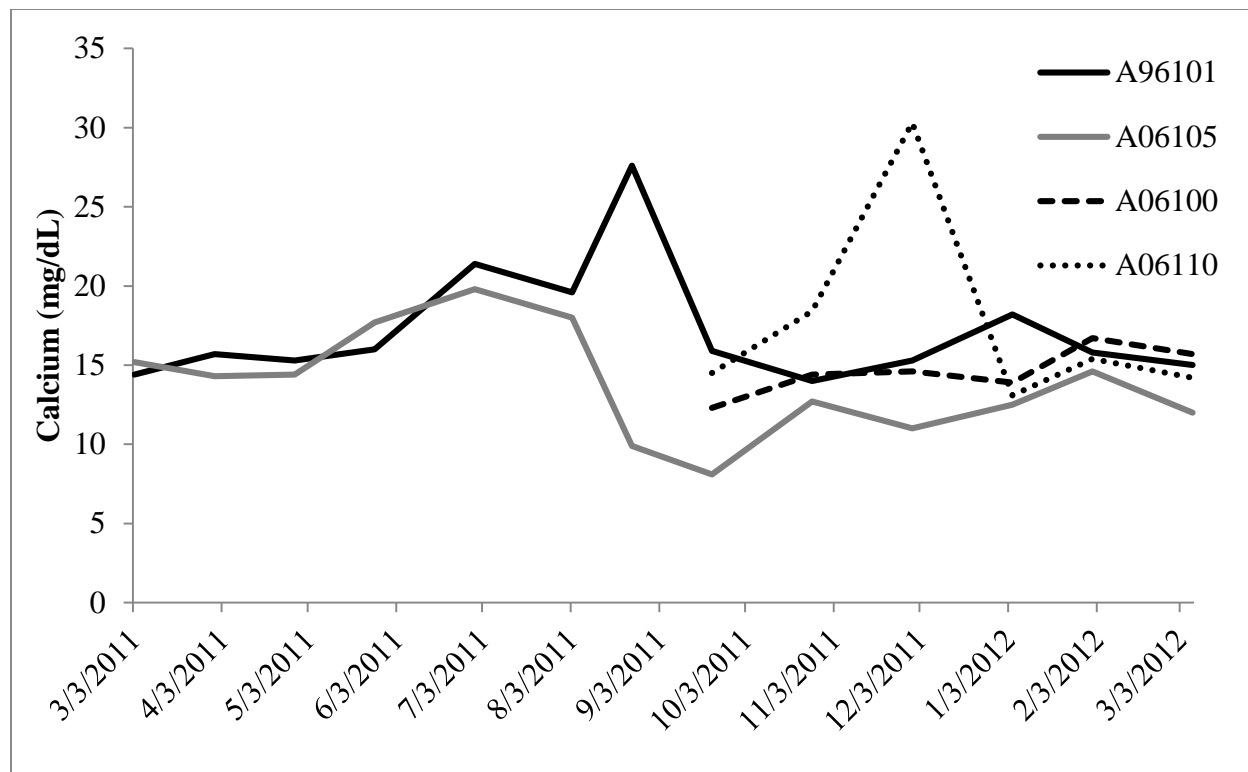


Figure 17- Individual calcium (mg/dL) levels of the four adult female Guatemalan beaded lizards in the study. Individual A06105 laid five eggs from September 23, 2011 to October 13, 2011 and A96101 laid five eggs from December 12, 2011 to December 25, 2011. Individuals A96101 and A06105 were available for the entire study, individuals A06100 and A06110 were sampled for the first time on September 21, 2011.

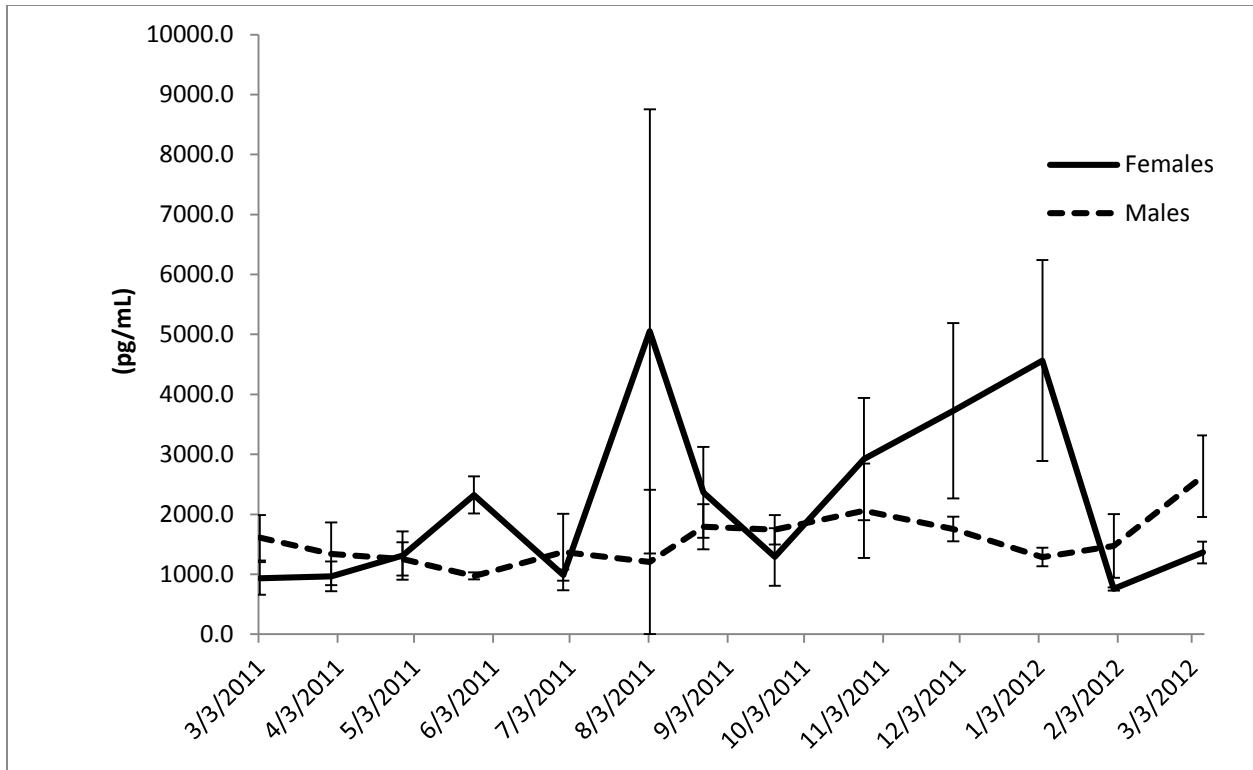


Figure 18- Mean monthly corticosterone levels of male and female Guatemalan beaded lizards. Values are means \pm standard error. Originally three males and two females were available for sampling. In September 2011 six males and two females were added to the study, for a total of nine males and four females.

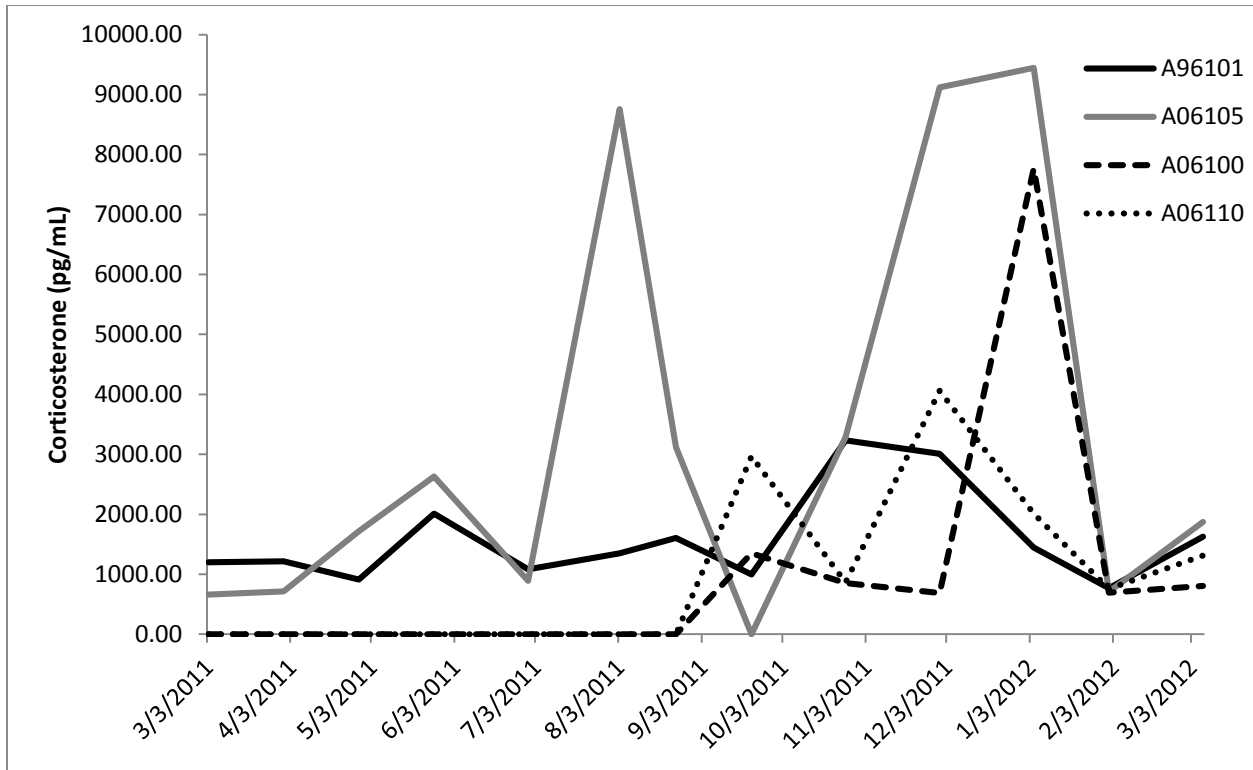


Figure 19- Individual corticosterone levels of the four adult female Guatemalan beaded lizards in the study. Individuals A96101 and A06105 were available for the entire study, individuals A06100 and A06110 were sampled for the first time on September 21, 2011.

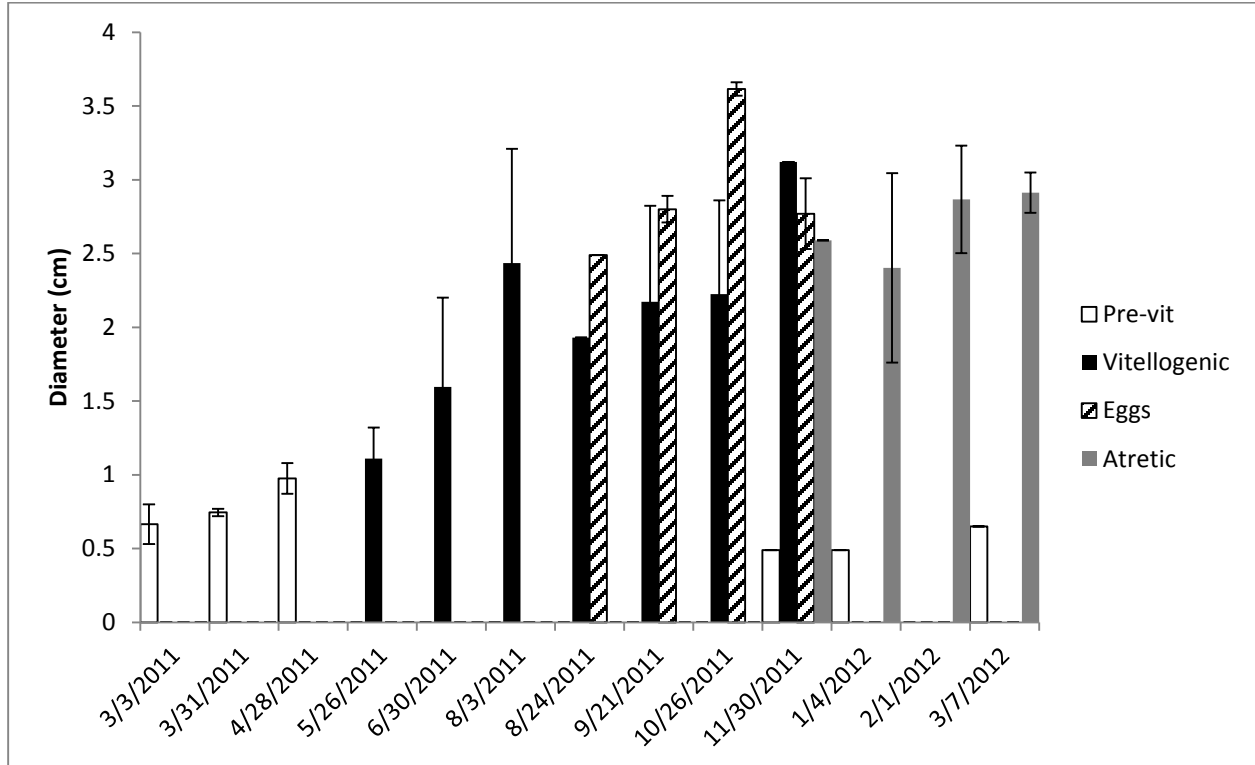


Figure 20- Average diameter of follicles and eggs in Guatemalan beaded lizards from March 2011 to March 2012. Follicular growth peaks during August through December when eggs begin to appear. Two individuals were sampled from March 2011 until September 2011, when two additional individuals were added (total of 4) from September 2011 through March 2012.

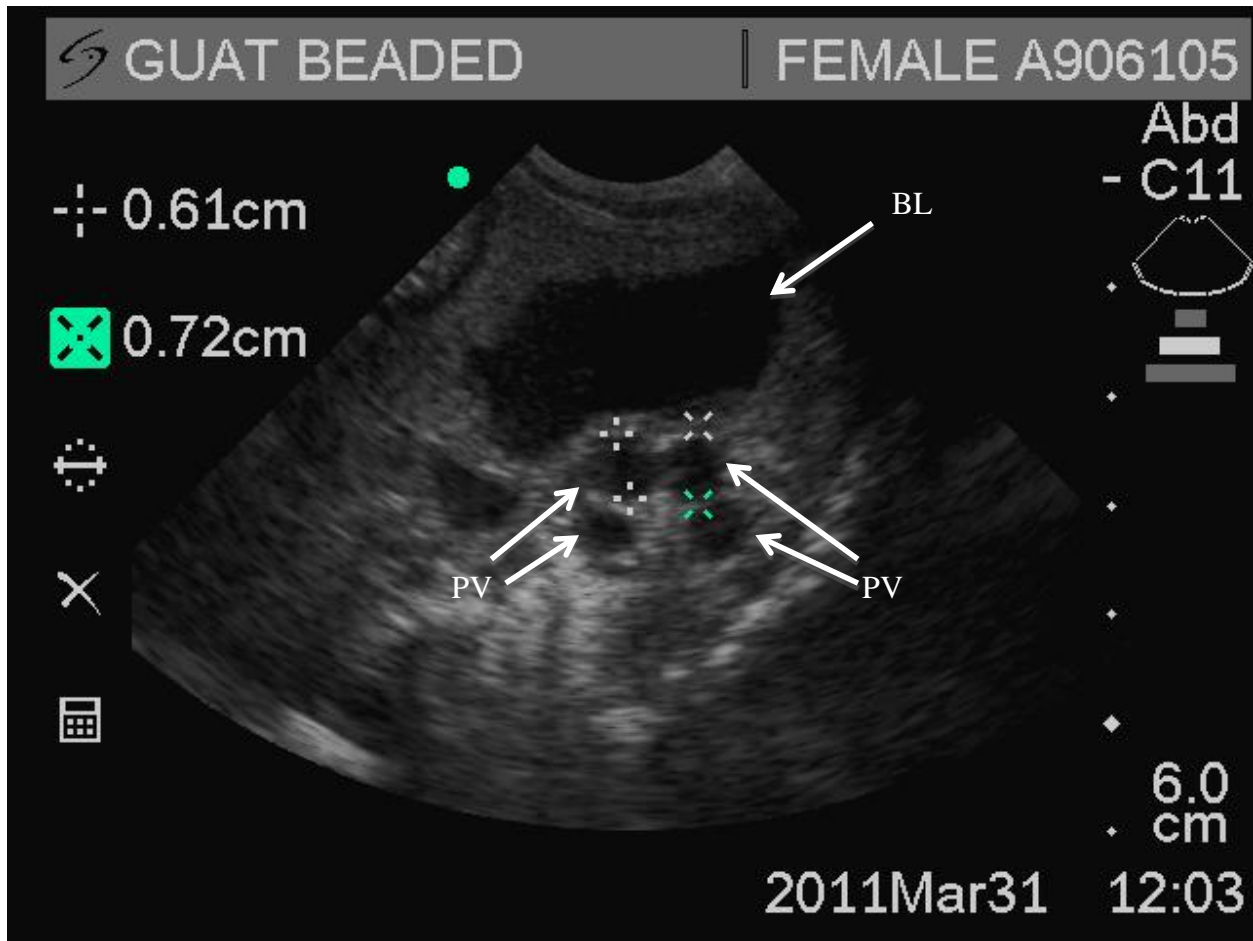


Figure 21- Ultrasound image taken of individual A06105 in March of 2011. Image is of four small previtellogenic follicles (PV). Two are measured with the ultrasounds internal calipers as 0.61 cm and 0.72 cm. The two measured follicles are indicated by cursors. The black appearance indicates previtellogenesis, as sound waves are passing through the follicle (anechoic). Bladder (BL) is also visible.

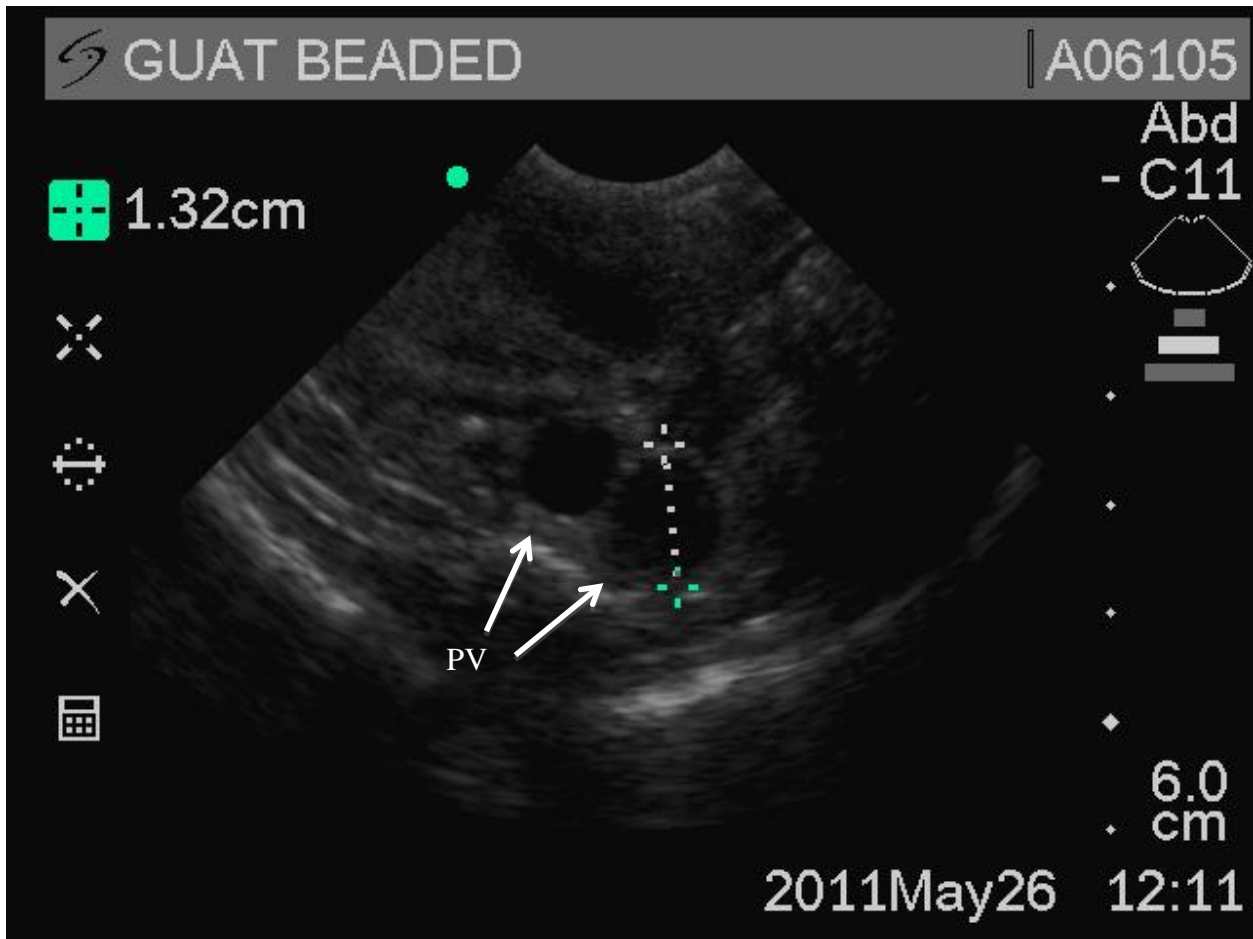


Figure 22- Ultrasound image taken of individual A06105 in May of 2011. Two previtellogenic follicles (PV) are present. The larger follicle is measured using the ultrasound units internal at 1.32 cm. The black appearance indicates previtellogenesis, as sound waves are passing through the follicle (anechoic).

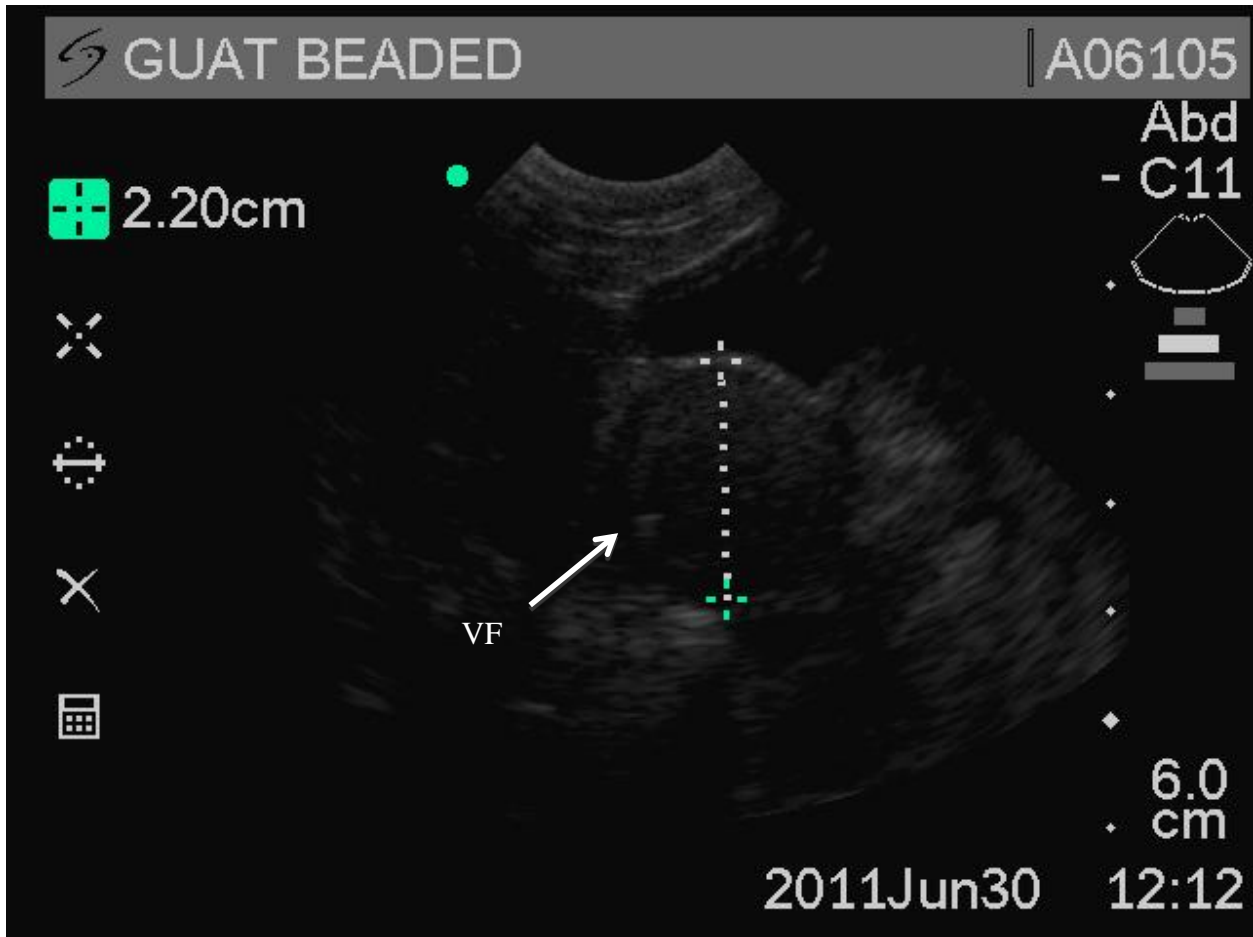


Figure 23- Ultrasound image taken of individual A06105 in June of 2011. One vitellogenic follicle (VF) is present. The follicle was measured using the internal calipers as 2.20 cm. Vitellogenesis is indicated by the grainy appearance, created by yolk platelets (vitellogenin) that are reflecting sound waves (echoic).

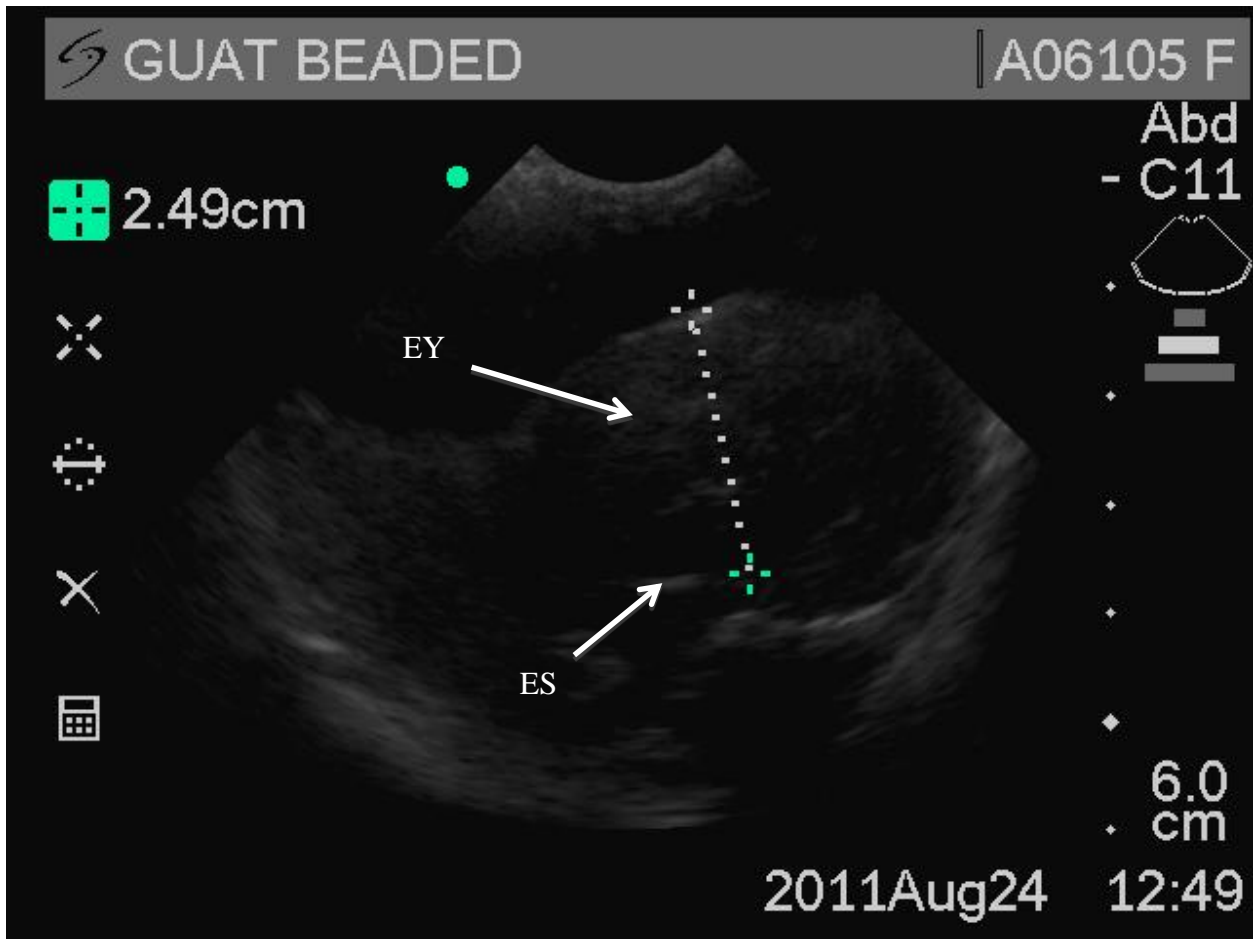


Figure 24- Ultrasound image taken of individual A06105 in August of 2011. One egg is imaged. The egg diameter is measured using the internal calipers as 2.49 cm. The white outline is the eggshell (ES) and interior grainy appearance is the egg yolk (EY).

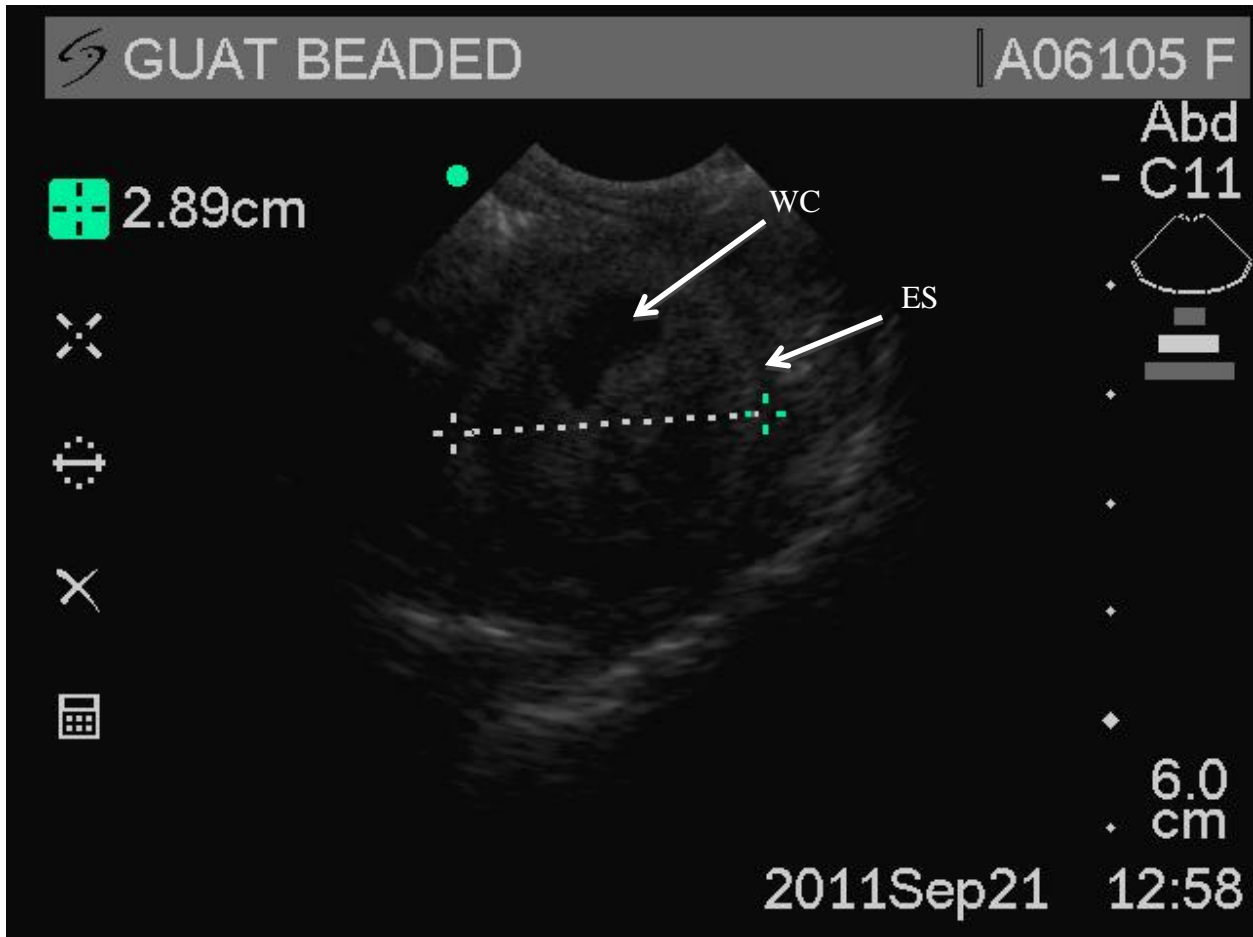


Figure 25- Ultrasound image taken of individual A06105 in September of 2011. One egg is imaged at a more developed stage than the egg seen in August 2011 (Figure 14). Development is indicated by water cavity (WC) (black area) in top portion of egg image.

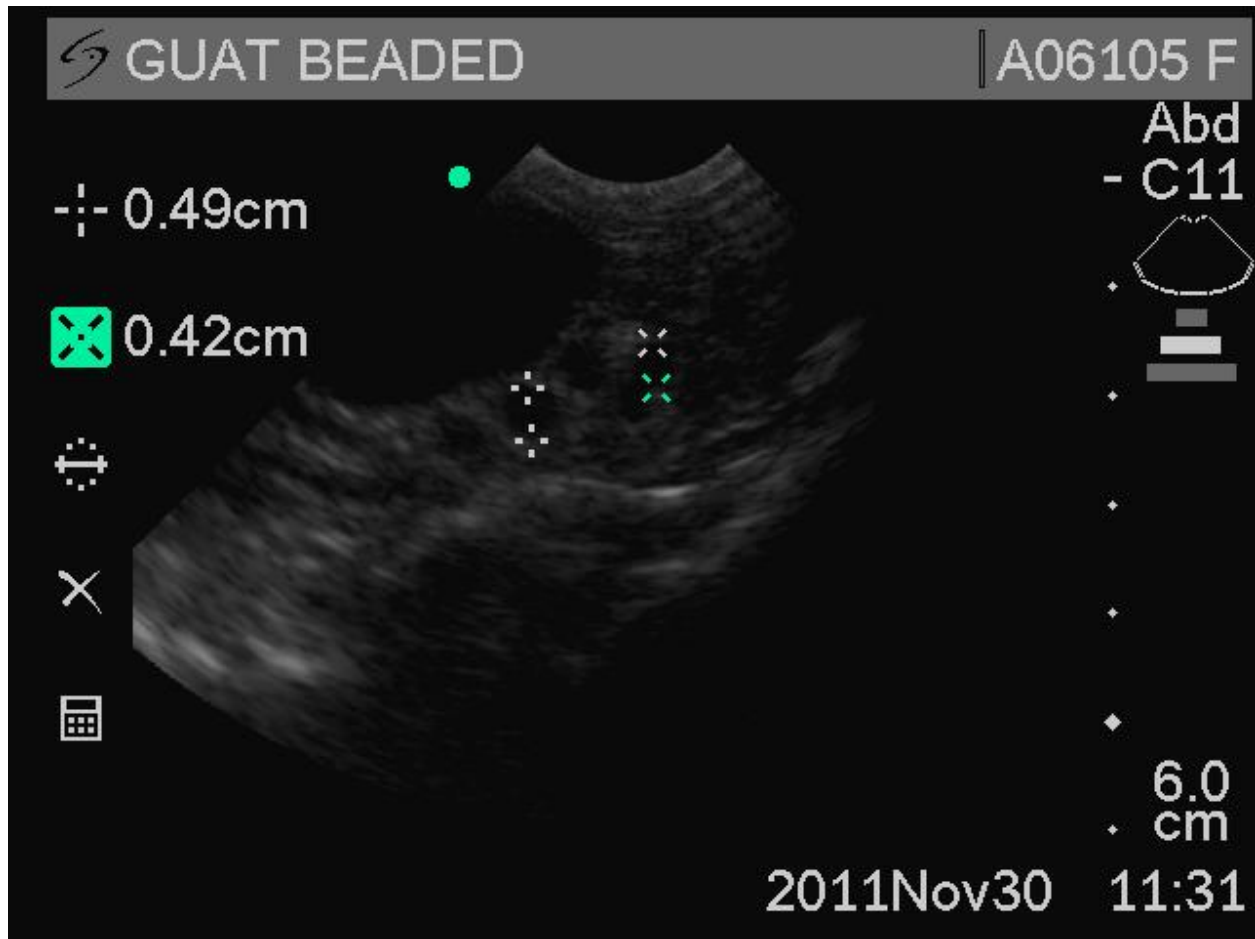


Figure 26- Ultrasound image taken of individual A06105 in November of 2011. Several small previtellogenic follicles are imaged. Eggs were laid on September 23, 2011. After oviposition two follicles were measured using the internal calipers at 0.42 cm and 0.49 cm. The black appearance indicates previtellogenesis, as sound waves are passing through the follicle (anechoic). These follicles will become next season's eggs.

DISSCUSSION

Reproductive cycle of the Guatemalan beaded lizard

The breeding group of Guatemalan beaded lizards at Zoo Atlanta displayed seasonal hormonal changes that coincided with the observed seasonal reproductive cycle. Testosterone in the male lizards' peaked during August indicating that breeding should be taking place at this time and shortly after during September and October. Testosterone peaks in males of lizard species correlate with maximum spermatogenesis and occur during breeding (Lovern 2011). Estradiol does not appear to cycle in a way that is easily observed the female lizards (Figure 12). While no cycle is discernable, ovulatory spikes are very well defined in two of the four females (Figure 14 & 15). Typically estradiol is elevated throughout vitellogenesis, which is not seen in Guatemalan beaded lizards. This could be attributed to a quick growth of follicles (3-4 months) as compared to some other species that can take several years to attain adequate follicular growth for fertilization, such as tuatara (Cree 2014).

Individual A06105 (produced one hatchling) had corresponding estradiol spike (Figure 15), calcium elevation (Figure 17), and follicular/egg development (Figures 21-26). Her estradiol spike was seen on August 3 (Figure 15) and eggs were first seen on ultrasound August 24 (Figure 24). Calcium levels were elevated during the months leading up to August 24 after which levels dropped (Figure 17). The first eggs were laid on September 23, 2011, two days after imaging a well developed egg with a water cavity (Figure 25). No other females produced viable eggs in 2011 and hormone and calcium levels were not correlated in other individuals. Estradiol spikes varied greatly over time. These spikes were associated with ultrasound data that was simultaneously collected throughout the study. It is possible that other spikes occurred, but were missed due to their short length and the frequency of sampling. Ovulatory spikes likely do not

persist for long periods of time (≤ 2 weeks); therefore with a sampling frequency of once a month, spikes could have been missed.

Calcium was higher in females than males and females had elevated levels of calcium during vitellogenesis, prior to egg production (Figure 16). Vitellogenesis and follicular growth correlate with increased calcium levels in several reptile species: Indian cobra, *Naja naja* (Lance 1976); painted turtle, *Chrysemys picta* (Callard et al. 1978); American alligator, *Alligator mississippiensis* (Lance et al. 1983); desert tortoise, *Gopherus agassizii* (Rostal et al. 1994); Kemp's ridley sea turtle, *Lepidochelys kempi* (Rostal 1991, Rostal et al. 1998); and tuatara, *Sphenodon punctatus* (Cree et al. 1991).

Corticosterone levels appear to increase in female Guatemalan beaded lizards during the egg laying season (Figures 18 & 19). Some species, such as tuatara (Cree 2014) and desert tortoise (Lance et al. 2001, Lance and Rostal 2002), have a natural corticosterone cycle similar to that of other steroid hormones (Tokarz and Summers 2011). In beaded lizards, corticosterone levels are similar in both males and females for most of the year. Individual A06105 showed signs of increased corticosterone starting in August for the 2011 reproductive season, earlier than other females. A06105 also showed follicular growth earlier than other females. Increased corticosterone levels are believed to be associated with mobilizing energy stores needed for nesting and these levels fall quickly after nesting (Cree 2014).

Ultrasound data shows follicular growth beginning in May and continuing through November when follicles are ovulated to become eggs or undergo atresia and begin to regress. This timing coincides with the testosterone cycle in male Guatemalan beaded lizards. Ultrasounds of male lizards were inconclusive, as no testes were observed, even during the months of August to October, when the testis should be at its largest size. Using ultrasound

technology also allowed us to further assist the zoo by confirming the sex of animals that arrived during the middle of the study. Sexing beaded lizards externally is quite difficult, typically males have a larger body and wider head, however this is not always true. A young individual (hatched in 2006), of approximately 500 grams, had follicular development at the same time as the four adult females. However, her follicles were smaller than those of other larger females in the study. Timing of hormone and reproductive cycles varied among the four adult female lizards. Individual variation is something to be expected; however, with so few individuals, these differences become more apparent.

Conservation of the Guatemalan beaded lizard

Tropical dry forests are seasonal with respect to rainfall, but not temperature (Beck 2005). Seasons of rainfall vary by location and are influenced by ocean currents and airflow patterns. The dry season in Motagua Valley can last up to eight months (November through June)(Beck 2005) with June to October being the rainy season, accounting for up to 80% of the annual rainfall (<500mm; Beck 2005). During the dry season there is very little food available, and wild caught animals are always thin (B. Lock pers. comm.). The rainy season causes impressive growth and food availability.

The few field observations of other beaded lizard species suggest that captive animals are on the same temporal cycles as wild populations (Goldberg and Lowe 1997, Alvarez del Toro 1982, Ramirez Velazquez and Guichard Romero 1989, Goldberg and Beck 2001, Beck 2005, Beck and Ramirez-Bautista 1991). Thus, based on what we see in captive Guatemalan beaded lizards, we expect that eggs would be laid as the dry season begins (October to December), after reproductive females had foraged extensively and gained in body condition during the preceding rainy season. As incubation takes up to 180 days, eggs in the wild would hatch as the following

wet season begins in May and food availability increases. This increased food availability would allow for excellent foraging opportunities for neonates. In captivity, eggs that come into direct contact with liquid die and become moldy, therefore it is believed that eggs must remain dry for proper incubation. Conditions in the wild during the time of incubation would allow for these dry type conditions that eggs in captivity appear to require.

The seasonal reproductive cycle of the Guatemalan beaded lizard is correlated with environmental conditions displayed in Figure 27. Seasonal temperature, day length, and rainfall are associated with physiological changes in male and female lizards. Female lizards begin to add nutrients and prepare follicles for reproduction during the wet season, when resources are plentiful. At the end of the growing season (August), follicles are at ovulatory size and ready to be fertilized. Males undergo spermatogenesis during the growing season as well, and are energetically prepared for any male-male competition that may ensue. After fertilization eggs are deposited from October to December, during the dry season. These eggs are believed to be laid in tunnel burrows and then back filled, rather than the traditional nests that are dug straight down (B. Lock pers. comms.). The eggs then incubate during the dry season for approximately six months. At the time of hatching the wet season is just beginning, allowing the neonates ample food availability.

Seasonality is seen in most animals, and requires that energy to be conserved for less optimal times. As the environment changes across the seasons so must the allocation of energy. Eggs are laid at a time so that when they hatch the offspring will have the greatest chance of success. Most reptiles do not display parental care after eggs are laid. Nest location and egg laying time (also egg hatching time) are the only way for the female to influence the chance of

success of her offspring. Seasonal reproduction is seen in many species of temperate zone lizards. In tropical regions rainfall and coincident food availability may favor seasonality.

Two females, A06105 and A06100, were one-two months earlier than others. This variation could possibly be attributed to the fact that A96101 is a captive bred animal, and much younger. Early reproductive seasons in a female reptile's life, of most species, often result in fewer, smaller, or incomplete production of eggs or follicles. Although she appears to be at a reproductive size, and is producing follicles, she may not be old enough to produce viable eggs. In our study, earlier egg laying meant that fertilization had occurred while animals were housed outside and animals had not been inside for very long before egg laying began. The outside enclosure provides more natural conditions for breeding. Outdoor pens provide accurate light cycles and temperature variations. Indoor conditions never mimic outdoor conditions perfectly. When outside, light and temperature slowly increase and decrease throughout the day and seasons. Rainfall happens naturally with all of the other environmental cues, including barometric pressure. These and other factors that we may not even know about, influence the physiology of animals.

Corticosterone levels vary throughout the year for different reasons, including stress. Short term stress can be beneficial because it helps with adverse situations requiring a fight or flight response (Ellenberg et al. 2007). Long term stress can be negative, resulting in decreased health, reduced fertility, and shortened life span (Ellenberg et al. 2007). Stressors can come from many different sources; human activity has been linked to elevated stress in free ranging species (Ellenberg et al. 2007). Hormones can affect embryos during development (Cree et al. 2003). In mammals, young that are born to a stressed mother do not perform as well, therefore it is beneficial for the female to mediate her stress response during pregnancy (Cree et al. 2003).

However, there have been an increase in the number of studies that have found a positive association between corticosterone levels and reproductive success (Moore and Jessop 2003). There is ample evidence for acute stress to decrease reproductive success, yet it is also evident that in some species there is a concurrent elevation in corticosterone during the reproductive season. It is possible that elevated corticosterone levels help with reproduction. Moderate elevations in corticosterone help to mobilize energy stores, which are needed during the reproductive season. There are two types of positive relationships between corticosterone and reproduction- seasonal elevation in corticosterone (weeks to months), or a shorter spike during pronounced energy expenditure (hours to days). There are several reptile species that have elevated corticosterone levels during the reproductive season: Side blotched lizard (*U. stansburiana*, Wilson and Wingfield 1992), Galapagos Tortoise (*Geochelone nigra*, Schramm et al. 1999), Red-sided garter snakes (*Thamnophis sirtalis parietalis*, Moore et al. 2001), male and female green sea turtles (*Chelonia mydas*, Jessop et al. 2002), gravid tuatara (*Sphenodon punctatus*, Tyrrel and Cree 1998), red-spotted garter snakes (*T.s. concinnus*, Moore et al. 2000). There are also species that have been shown to have no elevated corticosterone such as: brown anoles (*Anolis sagrei*, Tokarz et al. 1998), gopher tortoise (*Gopherus polyphemus*, Ott et al. 2000), female American alligators (*Alligator mississippiensis*, Guillette et al. 1997), and bearded dragons (*Pogona barbata*, Amey and Whittier 2000). There is evidence that female reptiles will still nest, even during stressful events, such as sea turtles seen nesting with recent shark attack (Jessop et al. 2004).

In the future, we would like to analyze other hormones such as progesterone and testosterone in the females. Because estradiol did not appear for longer periods at elevated levels during vitellogenesis, it did not provide a complete profile for the female lizards. Progesterone

could provide a more helpful profile. Progesterone is typically elevated during the gravid stage and slowly decreases after egg laying. Progesterone may be present in the plasma at higher basal levels, allowing for easier detection of the molecules. In certain groups, such as turtles, females have measureable circulating levels of testosterone. All females produce testosterone, however it is usually converted to estradiol before entering circulation. Data on other lizard species (Lovern 2011) indicates variation on which lizard species have circulating testosterone and which do not. Testosterone is seen in female tuatara and is thought to be associated with receptivity, and therefore could be a good indicator of when breeding should be happening (Cree 2014).

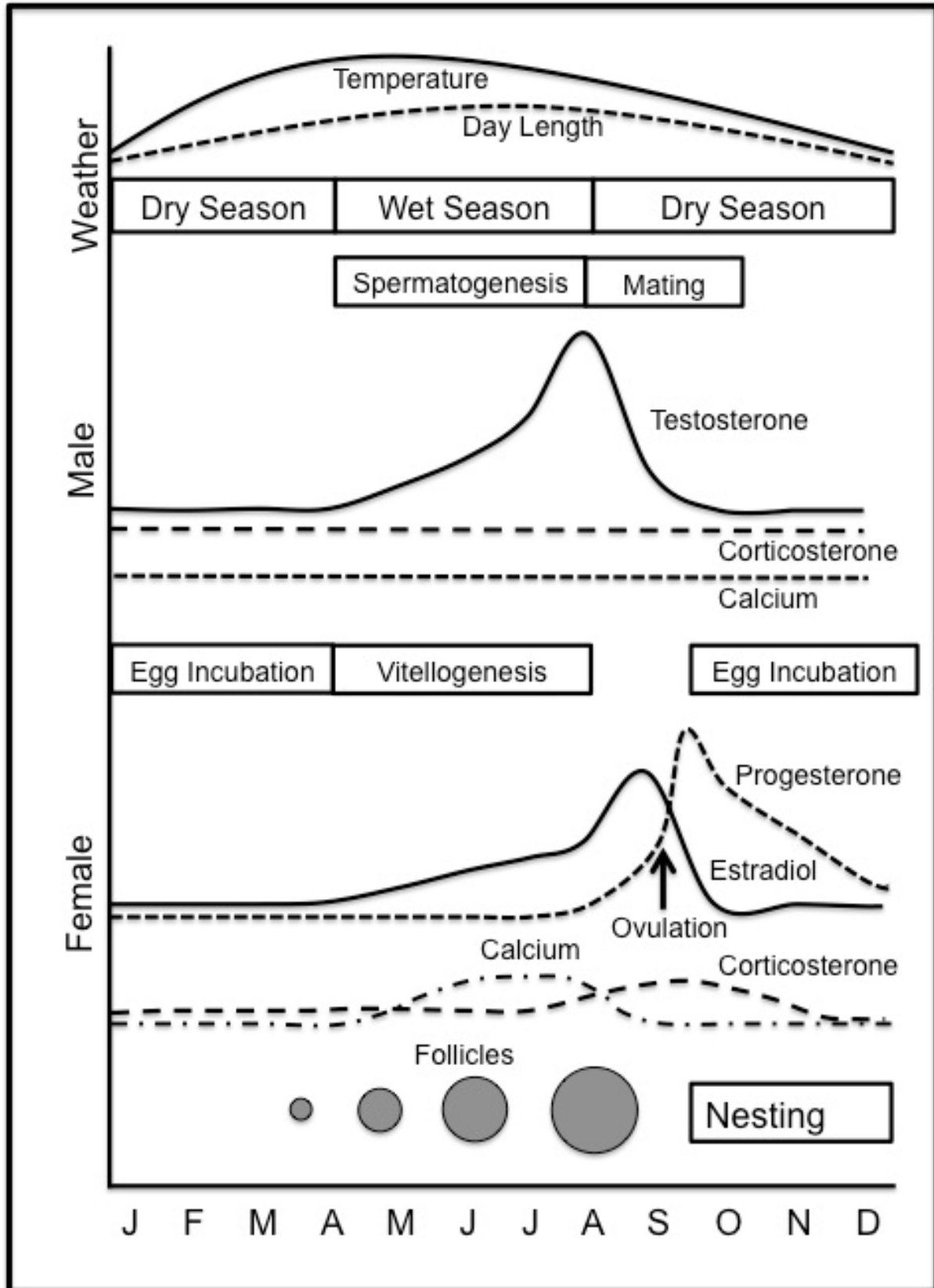


Figure 27- Conceptual schematic of reproductive cycle for Guatemalan beaded lizards, including weather, male cycle and female cycle. Temperature varies 5-6° C across the year. Day length varies 1.7 hours across the year. The wet season consists of 80% of yearly rainfall. Vitellogenesis occurs during wet season when food is plentiful for females. Egg incubation occurs during dry season and hatching happens at onset of wet season (with food availability).

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