SEX STEROID HORMONES,

ARCHITECTS OF BEHAVIOR AND DEVELOPMENT:

A LOOK AT THE EFFECTS OF SEX STEROID

HORMONES IN A LIZARD MODEL.

By

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Writing this dissertation and completing my research has been quite the ride. I came into grad school bright and shining, ready to learn and make an impact. Along the way I was caught with health issues and a global pandemic…and boy did that make research difficult. Nevertheless, I persevered, and thanks to my loving husband (then boyfriend) and the guidance of my advisor and advisory committee I was able to produce this dissertation. I can now embark on the world having gained much knowledge, and hopefully make that impact my former self aspired to.

Name: BROOKE M. EQUINOX

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Title of Study: SEX STEROID HORMONES, ARCHITECTS OF BEHAVIOR AND DEVELOPMENT. A LOOK AT THE EFFECTS OF SEX STEROID HORMONES IN A LIZARD MODEL.

Major Field: INTEGRATIVE BIOLOGY

Abstract: The mechanisms of sex determination and differentiation in reptilian groups are diverse and largely not well understood. With this study, we hoped to identify to what extent these mechanisms may be overridden by hormonal exposure, whether it be maternal embryonic, or exogenous in origin. We explored the extent of changes acquired by the offspring of *Anolis sagrei* treated with steroid hormones. Specifically, we documented how the introduction of estradiol (E_2) and testosterone (T) into the developing embryos of this species early in development affected not only physiology, but also behavioral patterns. In this study, we observed that E_2 and T affected many aspects of development. In our first project, we discovered that certain treatment methods for delivering hormones to females when testing maternal effects may be problematic. We specifically tested implantation as a delivery method for the hormones. After implantation, we discovered that oviposition had ceased for all groups, pointing to either a critical effect of stress or an interaction with application method. In this part of our project, we also observed that though oviposition ceased for all groups, our T treated females had a significantly higher number of yolking follicles than our controls. Similar results have been seen in hens and could apply to other reptiles if investigated in future studies. When we adjusted our study to directly treat eggs, we discovered that treatment of eggs with E_2 resulted in a female sex ratio bias. For those few resultant male offspring treated with E_2, we found that growth (in terms of snout-vent-length and mass) were significantly affected as hatchlings and at 30-days post-hatch. These males tended to be smaller than controls at these time-points, and as adults, they had significantly smaller dewlap-cartilage lengths. This suggests that E_2 plays a critical role in the developmental pathway for females and potentially leads to their smaller stature and dewlap lengths as adults. The major results of our behavioral study also identified E_2 as the major player. We found that E_2 treated females had on average higher aggression scores than controls indicating E_2 is involved in the organization of agonistic displays for female development. While we examined these hormones through the lens of approximating maternal and embryonic origin, future studies may want to approach these in terms of toxicological pollutants as many estrogenic mimics have been observed in habitats that are viable for this species.

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CHAPTER I

SEXUAL DEVELOPMENT: AN INTRODUCTION TO THE INVESTIGATION INTO THE CONSEQUENCES OF SEX STEROID HORMONES AND DEVELOPMENTAL TIME PERIOD

1.1 This Research

In this dissertation, I will explore the relationships between sex steroid hormones and sexual development at three different life stages of the Brown Anole (*Anolis sagrei)* – two during embryonic development and one in adulthood – with a glimpse into invasive species and environmental toxicology. The main questions of this research are: what time points of development are critical for sexual development, and what are the differing effects of Estradiol (E_2) and Testosterone (T) on this development? I predict that despite the time period of embryonic development, E_2 will have a feminizing effect on physiology and behavior, and T will have a masculinizing effect on physiology and behavior of adult Brown Anoles.

1.1.1 Anolis sagrei; Model Species and Invasive Species

A. sagrei, the Cuban brown anole, was the chosen model species for this study. It is an invasive species originating from Cuba, with its first invasion occurrence in the

United States occurring in the Florida Keys in 1887 (Garman 1887). Brown anoles became established in the southern United States in the 1940s, and by the 1980s, officially were considered invasive, a descriptive term that applies to wildlife that pose a threat to the environment, the economy, or human health and safety (Campbell and Echternacht, 2003). Since its original invasion, *A. sagrei* has spread to most southern states surrounding the Gulf of Mexico. The criteria met by *A. sagrei* to be considered invasive is its drastic takeover of habitat occupied by the native Green Anole, *Anolis carolinensis*, a very similar yet not closely related anoline species (Campbell and Echternacht, 2003).

Considering invasive species management, there are four goals that are set for management protocol aligning with the four stages of invasion: 1. Prevention of introduction of invasive exotic species, 2. Eradication of invasive species using the Early Detection and Rapid Response system, 3. Containment of the continued spread of invasive species, and 4. Reduction of established populations and maintaining them at the lowest levels feasible (Harvey and Mazzotti, 2014). For species that have made it to the fourth level of invasion, management is expensive and often ineffective. Gaining more understanding of invasive species can help to prevent further invasion and potentially aid in reducing their impact. In the world of hormones and behavior, much research has been conducted using *A. carolinensis* as a model species while less has been completed using *A. sagrei*. With this study, we hope to increase the information about *A. sagrei* so that we may have a better understanding of its physiology and potentially apply this to its future management.

1.1.2 Egg Synthesis and Hormones

The study of the implications of sex steroid hormones on a developing embryo is an exciting avenue of research. Due to the prolonged development that occurs in the shell of oviparous species outside of maternal influence, it has been assumed that maternal influence on offspring phenotype is limited or nonexistent when compared with viviparous species (Lovern and Wade 2001). In the last few decades however, we have seen experimental evidence of both experimentally elevated maternal steroids being transferred to eggs as well as naturally occurring elevated yolk steroids. In assessing the effects of maternal allocation of sex steroid hormones to offspring in oviparous species, it is also possible to study the effect to which embryos modify their hormonal environment due to the uncoupled nature of development.

Anoles are a great model for investigating the behavioral endocrinology of reproduction. They have a predictable breeding season that, in the U.S., begins in March/April, and ends in late August/September (Warner et al. 2009). Females are capable of sperm storage, requiring no other mating encounters after capture from the field. Females will lay single-egg clutches around every 7-10 days, making reproduction and egg collection predictable and frequent (Reedy et al. 2015). In addition, *A. sagrei,* like all anoles, is precocial, so maternal investment is completed at oviposition (Warner and Lovern 2014). Juveniles hatch from eggs around 4-6 weeks after oviposition, so early physiological effects may be seen in a relatively short span of time (Lovern et al. 2004a). In *A. sagrei*, egg production alternates between ovaries, meaning each egg is yolked and shelled independent of other eggs allowing maternal energy allocation among offspring to be individually controlled (Hall et al. 2018). This allocation has been seen in

experiments of maternal diet manipulation showing evidence of yolk T increasing with each egg females produce (Warner and Lovern 2014).

1.1.3 Embryonic Development

The embryological stages at which sex differentiation occurs in reptiles is generally between stage 14 and 22 of embryonic development in many taxa (Greenbaum and Carr 2001). During these stages, the gonad develops from an undifferentiated mass to either an ovary or testis, and consequently this developmental time period is also when gonadogenesis may be susceptible to hormonal manipulation (Greenbaum and Carr 2001).

Most of the embryological development of *A. sagrei* occurs post oviposition. Nineteen embryological developmental stages have been determined to exist for this species with oviposition occurring during stage four of development (Sanger et al. 2008). A more detailed look at developmental staging in reference to this study can be seen in Figure 1. The major sex differentiating events occur on average during days 9-13 of development, or stages 8-13 of the Sanger *Anolis* Development Model (Holmes and Wade 2005, Sanger et al. 2008). At day 9 (stages 8-10), gonads are undifferentiated. By day 10 (stage 10), gonads have the initial defining characteristics of either ovaries or testes. By day 13 (stages 11-13) ovaries have a well-developed cortex containing developing follicles, and testes have well-developed medullas with organized seminiferous tubules (Holmes and Wade 2005). Male and female brown anole embryos possess hemipenes, but in females, those hemipenes regress by incubation day 19. Treatment of female embryos on day 10 of incubation with T or dihydrotestosterone (DHT) leads to a regrowth or recurrence of prior hemipene structure, and treatment of

male embryos on day 10 of incubation with estrogens leads to a regression of hemipene structure (Holmes and Wade 2005).

1.1.4 Adult Hormones

Adult lizard hormones in seasonally breeding species such as *A. sagrei* tend to have large variation. In seasonally breeding males, T is generally highest at the time of year when they are exhibiting territorial and sexual behavior (Tokarz et al. 1998). Additionally, males in experiments that have elevated T levels tend to be more successful in defending their territory and have a larger territorial area (Tokarz et al. 1998). Additional evidence for variation of sex steroid hormones between seasons is that in the non-breeding season, when breeding behaviors decrease, the gonads become inactive, regress in size and weight, and decrease sex steroid production (Kang et al. 2020). In the related anole species, *Anolis carolinensis,* T is also a critical activator in courtship and aggressive behaviors, including elevated bite-force performance, but it is not the only sex steroid hormone that plays a role in adult behaviors. High levels of T coincide with elevated corticosteroid concentrations due to the energetically expensive cost of T (Husak et al. 2007). These elevated stress hormones can lead to lowered locomotor abilities and sexual signal quality (Husak et al. 2007). Estrogens in high levels, on the other hand, tend to result in increased female receptivity and facilitate mounting behavior in male green anoles (Wu et al. 1985, Wade 2011).

1.1.5 Objectives

In the chapters that follow, I examined the effects of embryonic steroid exposure on the differentiation, physiology, and behavior of brown anoles. In Chapter II, my objectives were to investigate the effects of maternally elevated T and E_2 on egg

production and oviposition. In Chapter III, my objectives were to examine the effects of T and E_2 on the sex ratio of *A. sagrei*. These two hormones were applied at two different time points to observe the relationships of maternal hormones and embryonic hormones. In Chapter IV, my objectives were to investigate the effects of T and E_2 on the behavior of adult *A. sagrei* when altered during one of two pre-ovipositional time periods.

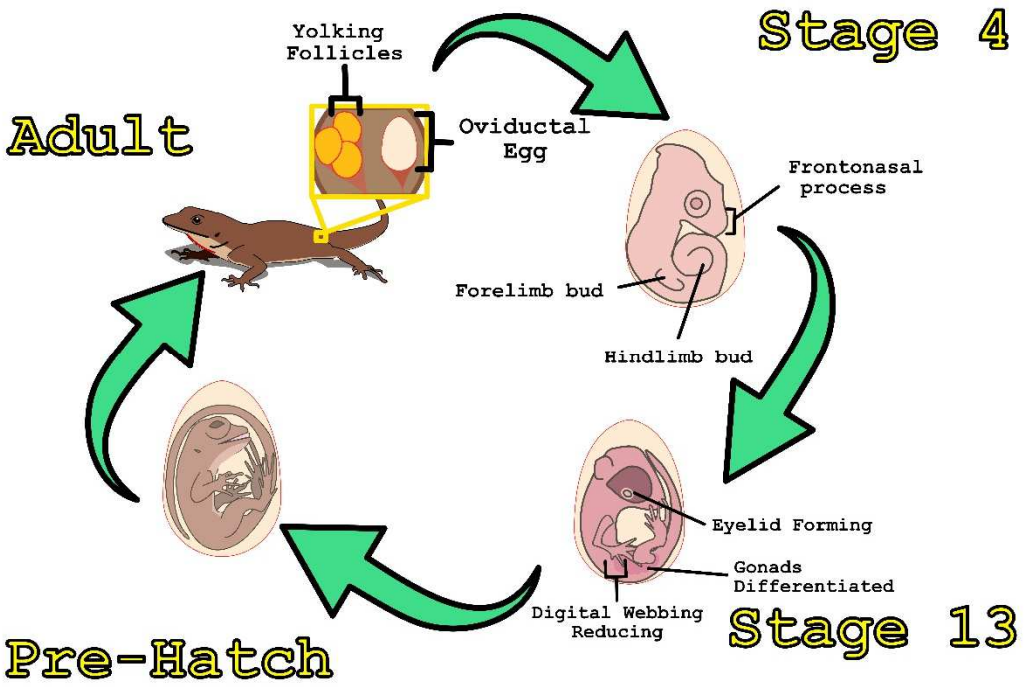

Figure 1: The major stages of development in reference to the effects of sex steroid hormones. At the adult stage, post-mating, females will begin allocating sex steroid hormones to yolking follicles, and from there begin shelling those yolking follicles from alternating ovaries every 7-10 days. Stage 4 is the Sanger stage of the embryo immediately after oviposition. Between stages 4 and 13, are when gonadal development occurs. By Stage 13, or day 15 of development, gonads are completely differentiated and external hemipenes for males are visible. Immediately pre-hatch, or day 28 of development, all features of the lizard are developed, including pigmentation.

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CHAPTER II

EFFECTS OF SEX STEROID HORMONES ON FOLLICLE PRODUCTION AND OVULATION IN A LIZARD MODEL: METHOD OF APPLICATION

2.1 Abstract

Sex steroid hormones such as testosterone (T) and 17β-estradiol (E_2) play important roles in development, reproduction, and behavior of many vertebrates, reptiles included. We investigated the potential for maternal effects that might occur in the lizard model *Anolis sagrei*, the Cuban Brown Anole. Since most studies of maternal effects in oviparous species are achieved with direct injection of hormones into eggs, we chose to simulate a more natural delivery method that approximates the way eggs naturally receive hormones through maternal allocation by treating mothers directly with implants. Implants were made by mixing Dow Corning silicone Type A medical adhesive with Sigma-Aldrich T, E_2, or left blank for controls. Oviposition rate was measured for 1 month prior to implantation, and 6 weeks post-implantation. The implantation procedure resulted in all females, including controls, ceasing oviposition. Females treated with T had significantly more yolking follicles in their ovaries compared Controls than E_2 treated females compared to controls. Thus, steroid exposure of T has an impact on the female reproductive cycle of the Cuban Brown Anole, and future studies should examine how implantation techniques affect oviposition rates of their model species.

2.2 Introduction

Sex steroid hormones have many functions, regulating everything from physiological development to behavior. These hormones go through different cycles of concentration during organismal life history, fluctuating with, for example, age, sex, the time of day, or even the season. For example, in female *Alligator mississippiensis*, Estradiol-17β (E_2) and Testosterone (T) increase prior to and during the breeding season (Gans and Crews 1992). In female *Anolis carolinensis*, estradiol increases as yolking follicles reach peak size (Gans and Crews 1992). These fluctuating levels of sex steroid hormones can be differentially allocated into the yolk of developing eggs (Gans and Crews 1992). Therefore, given that hormones are allocated into egg yolk and that allocation can vary with a variety of endogenous and environmental features, oviparous species can be great models with which to study maternal effects.

In many studies testing maternal effects of exogenous hormones on oviparous species, hormones are injected into an egg directly (Andersson et al. 2004; Sockman and Schwabl 2000). A potentially more natural way of testing maternal effects of exogenous steroid hormones would be to give reproductively active females hormone implants. These implants should increase the circulating hormone levels available in the mother giving a greater chance to allocate these to developing offspring, thus approximating the natural way by which yolk accumulates steroid hormones (Janzen et al. 2002). There can be drawbacks with this method, however. Females may reduce or cease egg production with introduction of these hormones. For example, application of exogenous T via injection resulted in lower total clutch size in zebra finches (Rutkowska et al. 2005).

Similar effects were seen in dark-eyed juncos with experimentally elevated T. These females laid significantly fewer eggs when compared to the control treatment (Gerlach and Ketterson 2013). Given that reproductive steroids play a critical, central role in reproductive physiology, it is no surprise that manipulating steroid concentrations can influence reproductive output altogether. The goal, then, for those wishing to study effects of yolk steroid allocation is to be able to sufficiently alter sex steroid concentrations such that differences among treatments are detectable, but not so much that reproduction significantly slows or ceases altogether.

The present study tests the effects of sex steroid hormones, specifically T and E_2, on follicle and egg production in anoles. Using the Cuban Brown Anole (*Anolis sagrei*), we hypothesize that hormonal treatment groups will not have a different average follicle and egg production than the control groups. *A. sagrei* generally follow a seasonal breeding pattern beginning in April and ending in late August/September (Warner et al. 2009). Mating continues throughout the breeding season, but females are able to store sperm for later fertilization. Females will lay single-egg clutches every 10 days or so (Reedy et al. 2015). In general eggs are oviposited from alternating ovaries (Lovern and Passek 2002) and buried in damp substrate, after which no further parental care is provided. If rate of oviposition is equal across groups prior to implantation, post-implantation rate of oviposition should be comparable if the dosage chosen has not affected the females.

2.3 Methods

Procedures described below were approved by the OSU IACUC (AS-17-8).

2.3.1 Animals and Treatment Groups

Fifty field-collected *Anolis sagrei* females (age range unknown, but all adults) were measured for snout-vent-length (SVL) to the nearest mm and mass to the nearest 0.01 g. Females were then randomly separated into three treatment groups: Control (blank) (n=17), E_2 (n=16), and T (n=19). They were then placed into 29-gallon aquaria with 3-5 females per tank with each tank holding females of a single treatment type. Females were purchased through Underground Reptiles (Deerfield Beach, FL). Treatment groups were given subcutaneous implants through injection (details below).

2.3.2 Animal Husbandry

Females were housed under laboratory conditions known to stimulate egg production. These conditions included a 14:10 hour light:dark cycle with n cage temperature of 28-38º C during the 14 "daylight" hours (hot and cool tank regions relative to the position of the basking light) and approximately 18º C during the "nighttime" hours (Lovern and Wade 2001). Cages contained perches, hides, water dishes, a peat moss substrate, and nest boxes containing dampened peat moss for egg-laying. Females had access to water *ad libitum* and cages were additionally sprayed with water daily. Females were fed 3-4 times per week with mealworms, wax worms, and/or vitamin- and mineral-dusted crickets (Herptivite and MinerAll with D3, respectively).

Nest boxes also were checked 3-4 times per week for the presence of eggs for 4 week prior to implantation and 6 weeks post implantation.

2.3.3 Implant Protocol

Implants were made following a modified protocol of Lovern et al. (2004b.). Briefly, ~700-800 mg silicone Type A medical adhesive (Dow Corning) was mixed with ~45-50 mg of E2 or T (Sigma-Aldrich) for treatment groups, or left blank for control groups. The silicone mixture was then reformed into ~1 mm diameter strings (to be cut into ~2 mm segments) by expelling it through a 3cc syringe. Total dosage for implants was ~0.656 mg for E2 implants and ~0.594 mg for T implants. These are comparatively low-dose implants (Cox et al. 2009).

Females were anesthetized via inhalation with isoflurane. The incision site was then sterilized using chlorohexane antiseptic while the implant was sterilized using sporox. Implants were injected subcutaneously and dorsolaterally using a pit tagger and then the incision site was sealed using Vetbond tissue adhesive (3M). Implants have a slow release effect and last ~4-6 weeks (Lovern, unpublished data). Incision sites were checked regularly to see if implant replacement was necessary.

2.3.4 Euthanization and Final Animal Processing

After egg production had ceased for 6 weeks, we quantified the final snout-vent-length (SVL) and mass for each female. We then euthanized all females (n=40) via isoflurane anesthesia overdose followed by rapid decapitation. We conducted necropsies

to quantify follicular and shelled, unlaid egg count. An incision was made on the ventral side of the body going through the cloaca up to the center of the chest between the forelimbs to make the ovaries visible. We recorded the number of developing follicles and shelled eggs in the reproductive tracts of each female. These counts were taken by hand. Figure 2 shows the yolking follicles found in the ovaries of one T treated female.

2.3.5 Data Analyses

Total output of eggs and yolking follicles were compared across treatment groups (E_2, T, and Blank (Control)) using one-way ANOVAs followed by Tukey *post hoc* tests when overall *P*-values were ≤ 0.05. Relationships between maternal traits (SVL and body mass) were ruled out by random assignment of treatment group and a one-way ANOVA comparing total egg count of each treatment group prior to treatment.

2.4 Results

2.4.1 Pre and Post-implant Egg Count

Prior to implantation, the rate of oviposition was equal for individuals of all treatment groups (T, E_2, and Blank or Control) (One-way ANOVA $F_{2,10} = 0.002$, $p > 0.05$). All three treatment groups produced a similar mean egg count prior to treatment (Figure 3). Immediately post-treatment and for six weeks after, all treatment groups ceased oviposition (including the control group), but not production of eggs.

2.4.2 Average Number of Yolking Follicles in Females

After hormone implants, we counted the number of developing follicles and shelled eggs that were present in the ovaries. We compared the average number of yolking follicles in the reproductive tract of each individual for the three hormone treatment groups. There was a significant effect of Hormone Treatment on the average number of yolking follicles for the three conditions (One-way ANOVA: Hormone Treatment, $F_{2,37} = 12.09$, $p < .001$). Post-hoc comparisons using the Tukey HSD test indicated that the mean count for individuals with T implants ($\overline{x} = 3.06$, SD = 1.57) was significantly lower than both the E_2 implant ($\overline{x} = 1.56$, SD = 1.13) and blank implant ($\overline{x} = 1.00$, SD = .66) groups. The E_2 implant group and blank implant group did not differ significantly from each other (Figure 4). Whereas, T implants led to significantly more yolking follicles, there was not a significant effect of Hormone Treatment on the number of shelled eggs produced. We tested the effect of Hormone Treatment on the number of shelled eggs found in the reproductive tract of individuals and found that there was no difference between groups (One-way ANOVA: Hormone Treatment $F_{2,37} = 1.08$, $p > 0.05$). The E_2 implant group had no shelled eggs in the oviducts (Figure 5).

2.5 Discussion

2.5.1 Treatment Procedures

Currently, there is no standard method for studying maternal effects of exogenous hormones in oviparous reptiles. Each method has its own pros and cons. Implantation allows for a slow release of hormones over a long time-period, but is an invasive

procedure (Gerlach and Ketterson 2013). The method of dermal absorbance of hormones is non-invasive, but requires frequent disturbance of individuals for continued application (Breuner et al. 1998; Knapp and Moore 1997). Direct treatment of food is another non-invasive method (Breuner et al. 1998), however, the exact amount of hormones cannot be controlled due to non-equal access to food in group housing, or failure of an individual to eat. Direct injection of eggs is simple and results in few deaths, but is seen as an unnatural method of hormone transfer (Andersson et. al, 2004). Filling this gap in the research field will greatly improve the effectiveness of treatment, while also reducing waste of animals and materials.

2.5.2 Implants and Their Effects in Anolis sagrei

In this study, we investigated implantation as a source for elevating maternal hormone levels. Egg counts were taken prior to and post implantation via daily egg collection and necropsy following euthanization. Implantation had a surprising result on oviposition. All individuals laid eggs at a normal rate prior to implantation (Figure 3). However, all egg laying ceased for all groups post-implantation. This is likely an effect of stress as even control females ceased oviposition (though we did not directly collect measurements of stress). Similar effects have been observed in a study on hens wherein oviposition was delayed when hens were moved into a new cage with unfamiliar conspecifics, which was quantified to be a stressful event (Reynard and Savory 1999). An additional study which examined heat stress on Shelducks presented similar results of a decrease in oviposition (Ma et al. 2014).

With so many cases of stress affecting egg production we theorize that this cease in oviposition post-implantation was due to an element of the procedure such as the implant material or the anesthesia used. This anesthetic, while suitable for rats, may not be suitable for reptiles (Smith et al. 2004). Females should have still produced eggs as it was still early in the breeding season. But for the individuals given T or even E_2 implants, this could additionally be a result of increased steroid levels (Gerlach and Ketterson 2013; Navara et al. 2015; Rutkowska et. al, 2005). We tried to use low-doses in our implants; possibly even lower doses are necessary but this runs the risk of not producing enough steroid release to be able to detect its presence in blood or yolk and future studies may want to measure if the dose we chose could be detected in the blood plasma of treated individuals.

Though oviposition ceased, egg production did not. Elevating T and E_2 levels in fertilized female *Anolis sagrei* lizards significantly affected egg production. Females given T implants, as compared to the E_2 and Blank treatment groups, significantly increased eggs in their reproductive tracts and also appeared to show larger yolking follicles (personal observation of size). As most studies do not look at the number of eggs in the reproductive tracts of individuals, there is little reported on this in the literature. One study, however, did note that increases in T resulted in a significantly increased rate of internal ovulation for hens (Navara et al. 2015). Comparatively, individuals given blank or control implants in our study seemed to have significantly lower egg production after implantation. Another explanation for this increase in yolking follicles for T treated females is that in many species, T concentrations increase around ovulation suggesting that it plays a role in female egg production (Rhen et al. 2002; Weiss et al. 2002). These

individuals had the expected number of follicles remaining, suggesting there was no reabsorption of eggs following the cease in oviposition.

In the future, we would like to test other methods, such as dermal absorption of hormones using sesame oil as a vehicle applied directly to the ventral surface of our F1 generation females above the ovaries. Using this non-invasive method, we can treat the eggs directly allowing for controlled application of the hormones in a more natural manner without compromising sample size due to reduced oviposition that may result from a stressful surgical procedure. We would also suggest that future studies examine which variable in our implantation procedure was the cause for the cease in oviposition. We recommend three main variables as the cause for this cease in oviposition: isoflurane anesthesia, sporox as the implant antiseptic, and stress. Testing for the effects of isoflurane anesthesia on oviposition rates would be the first approach we would recommend investigating.

Figure 2: A: four developing follicles in the right ovary of a female *Anolis sagrei* lizard. B: three yolking follicles in the left ovary of an *A. sagrei* lizard.

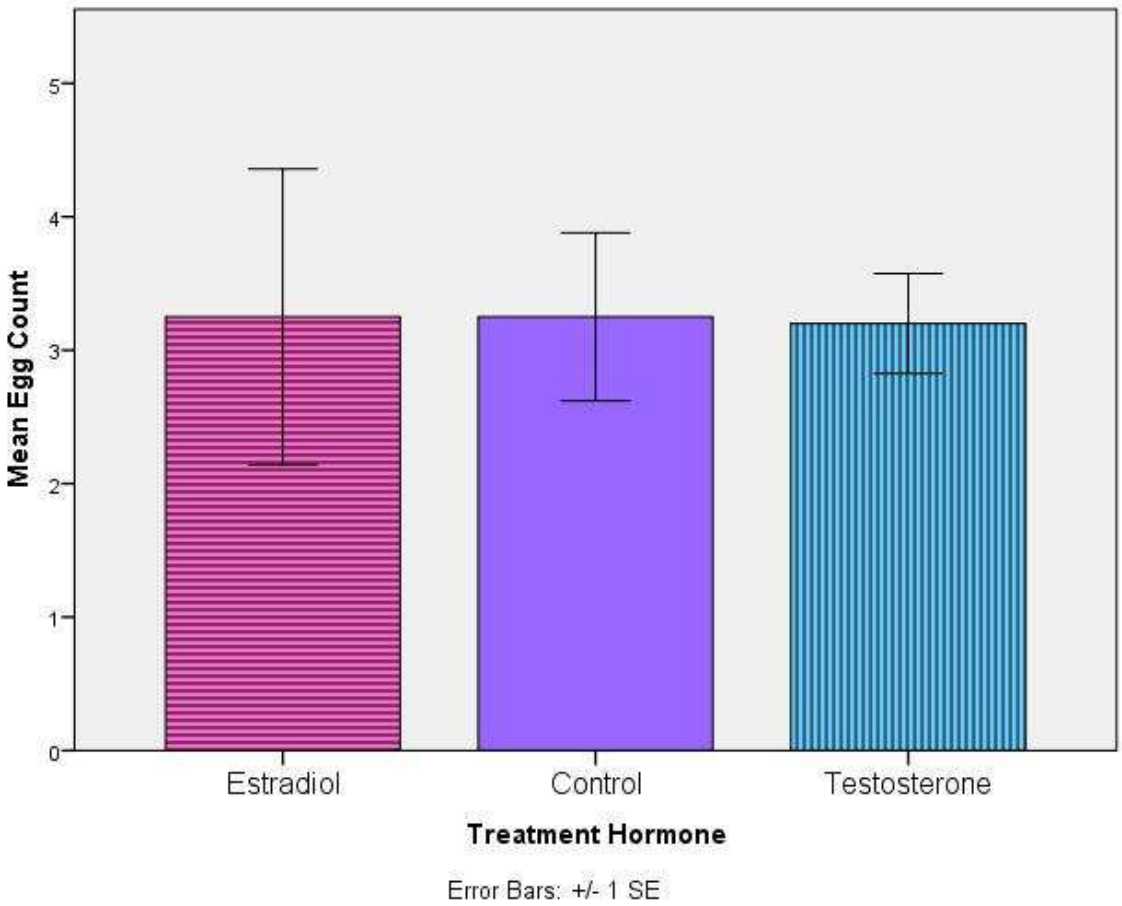

Figure 3: Female *Anolis sagrei* lizards showed equal number of eggs oviposited pre-implant for each treatment group.Data are presented as means ± SE.

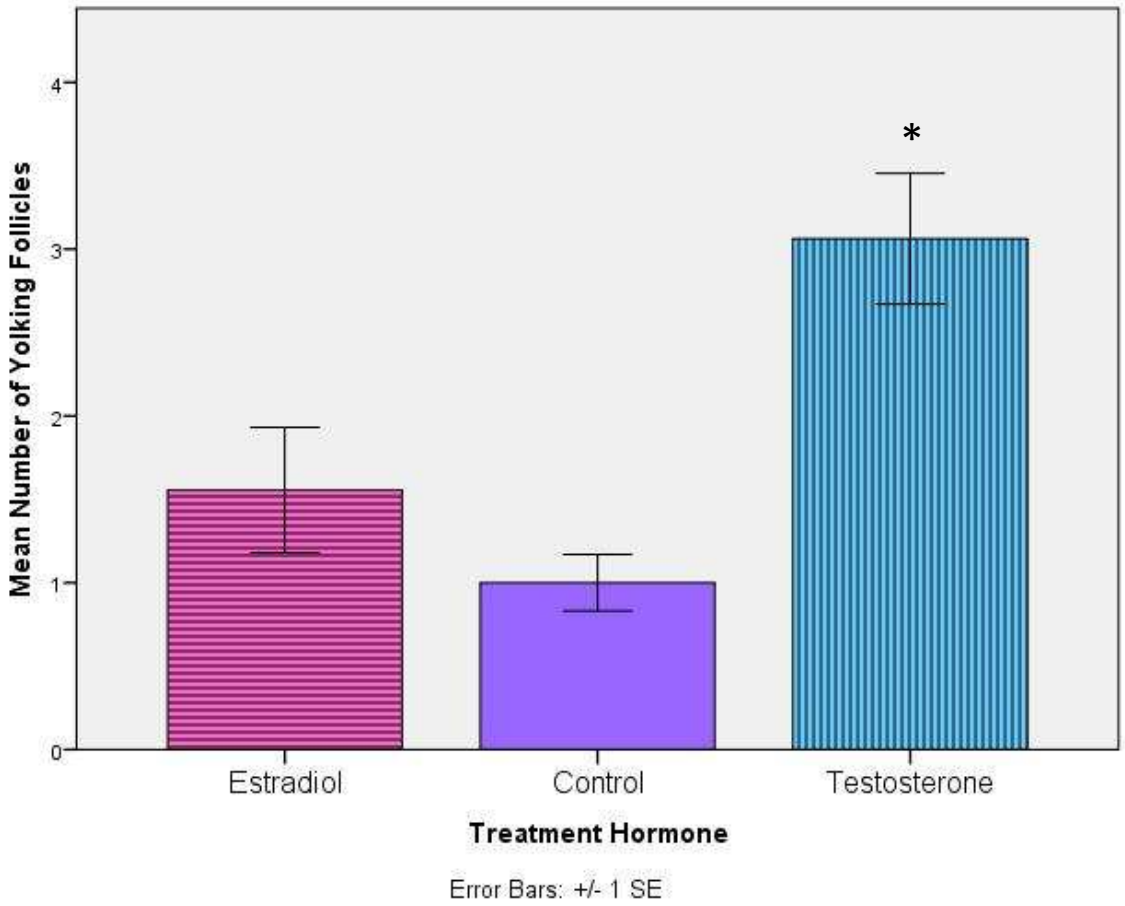

Figure 4: The number of yolking follicles present in the ovaries of female *Anolis sagrei* lizards by treatment groups. The Testosterone treatment group showed significantly more yolking follicles, but Estradiol and Control groups did not differ from one another. An asterisk indicates a significant difference ($p < 0.05$) Data are presented as means ± SE.

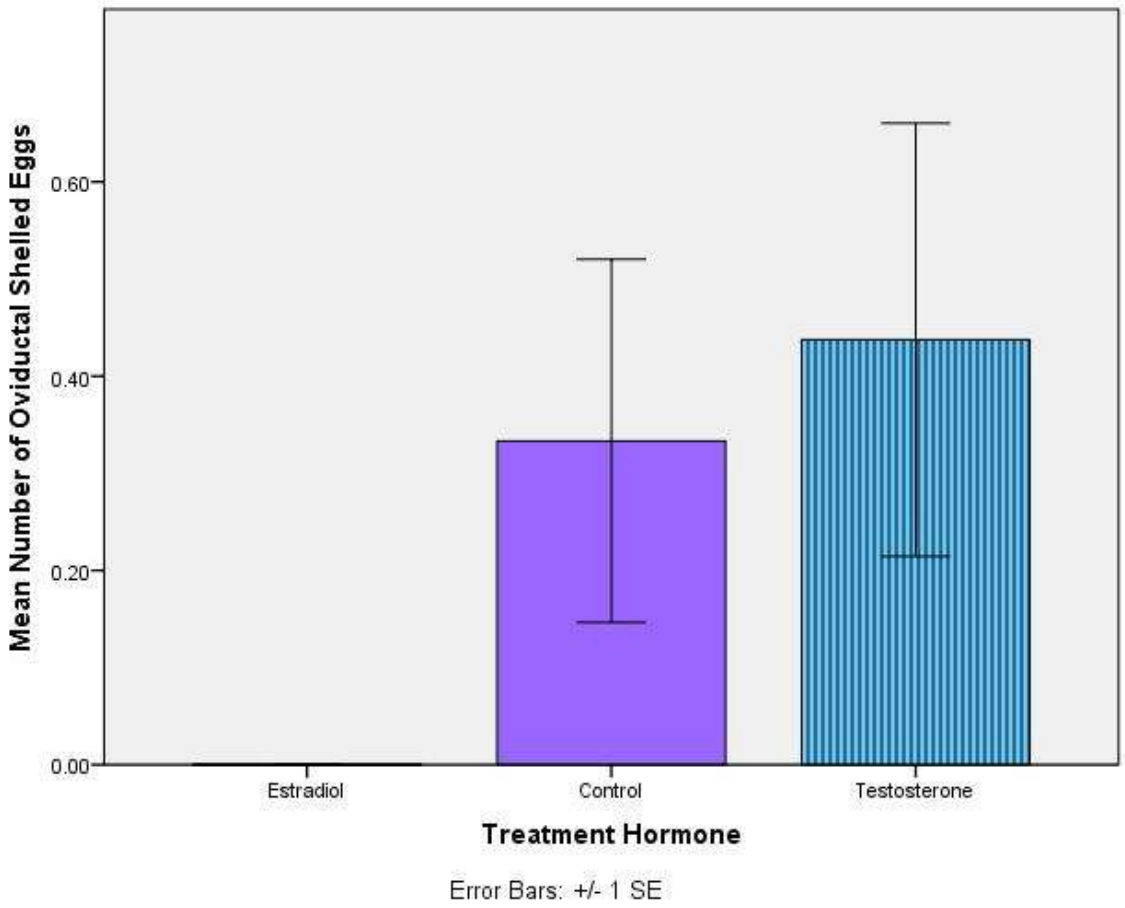

Figure 5: The number of shelled eggs present in the oviducts of female *Anolis sagrei* lizards was not significantly different between Testosterone and Control treatment groups. The estradiol treatment group had no shelled eggs in the oviducts. Data are presented as means ± SE.

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CHAPTER III

SEX REVERSAL AND THE EFFECTS OF SEX STEROID HORMONES ON THE PHYSIOLOGY OF DEVELOPING *ANOLIS SAGREI*

3.1 Abstract

Sex reversal mediated by sex steroid hormones has been seen in many taxonomic groups including reptiles. Most reptilian studies however have looked at species that exhibit temperature-dependent sex determination. We examined the effects of early administration of sex steroid hormones on a species with genotypic sex determination, *Anolis sagrei*, the Cuban Brown Anole, which is an invasive anole species in the United States. To test such effects, we applied Estradiol (E_2) or Testosterone (T) at two time points of development directly onto the eggs and examined the resulting sex and physiology. We observed that E_2 treated eggs were statistically more likely to develop into females compared to controls than T treated eggs compared to controls. This indicates that E_2 is an important sex steroid hormone in the pathway for female development in *A. sagrei.* After individuals had been raised to adult age (~1 year of age) we examined adult physiology for differences. We discovered that hormone treatment had a significant effect on male dewlap cartilage length, with males treated with T having significantly smaller dewlap cartilage lengths and left testis weights compared to controls

than the E_2 treated males. This result is surprising as many studies attribute high T levels in anoles to greater success in territorial defense which includes frequent dewlap-extension displays and higher fitness for those of larger size. Future studies should look at the impact that an increased female to male ratio has on the invasion success of this species, as well as the impact on reproductive success of resultant males from exposed eggs.

3.2 Introduction

3.2.1 An Introduction to Sexual Determination

Sex can be defined at multiple biological levels—behavioral sex, morphological sex, neurological sex, and genotypic sex, for example. Additionally, the mode of sex determination itself can vary. There are, in general, two different methods of sex determination that have been investigated; these are genotypic sex determination (GSD) and environmental sex determination (ESD). Over the past few decades, there have been many studies over sexual differentiation and sex determination. However, the majority of studies examined these phenomena in species that exhibit temperature-dependent sex determination (TSD), a type of ESD. In reptiles, the method of sex determination varies, with different methods sometimes found within the same genus (Rovatsos et al. 2014, Quinn et al. 2007). For example, Agamidae and Eublepharidae are two lizard families wherein GSD and TSD both appear (Rhen and Schroder 2010). Another example would be two species of turtles, the Wood Turtle (*Clemmys insculpta*), which has GSD and another member of the genus, *Clemmys guttata*, that exhibits TSD (Ewert and Nelson 1991).

It is even possible that a single species may exhibit both genotypic and environmental sex determination, for example in some reptiles that undergo sex reversal due to extreme temperatures during incubation (Quinn et al. 2007). One such species is *Basianna duperreyi*, a scincid lizard. This lizard has GSD that can be overridden by temperature when eggs are incubated at cool temperatures leading to an overproduction of males (Radder and Shine 2007). Another example of a lizard that has exhibited sex reversal from genotypic to environmental sex determination is *Pogona vitticeps*, the Australian Bearded Dragon. This lizard has haploid sex chromosomes where males are genetically ZZ and females are ZW (similar to the system found in birds). There has, however, been documentation of ZZ females found in the wild that were formed due to a temperature override (Holleley et al. 2015). More recent research has revealed yet another lizard, *Crotaphytus collaris*, (which primarily has GSD), exhibiting a temperature override of sex determination (Santoyo-Brito et al. 2017). In this species it has been noted that genotypic female eggs will result in males when incubated at extreme temperatures, and it has been theorized that females will select nest sites without temperature interference so that GSD will function (Wiggins et al. 2020).

The events that influence sex determination—both environmental and genotypic—occur during the early stages of embryonic development and trigger a hormonal cascade to initiate sexual differentiation (Greenbaum and Carr, 2001). Sex determination is defined by the molecular events dictated by a master regulator that is either a gene or environmental regulator (Gamble 2019). This master regulator directs whether the embryo will begin the development of male or female gonads. Sex determination sets the stage for sexual differentiation to begin. Sexual differentiation can

then be defined as the response to gonadal hormones and development of primary and secondary sex characteristics (Greenbaum and Carr, 2001). These events whether controlled by a specific gene—such as SRY in mammals—or environmental factors, are the first step towards defining the sex of an individual organism.

Within reptiles, there is considerable variation in the types of sex chromosomes found. Some have the typical mammalian XX/XY sex chromosomes in which males are the heterogametic sex, or avian-typical ZW/ZZ sex chromosomes in which females are heterogametic, but the majority of reptiles studied to date have atypical sex chromosomes. These atypical sex chromosomes are characterized by either not being morphologically distinct from each other (homomorphic) or by containing more or less than two, e.g. XXY (heteromorphic; Gamble et al. 2013). For *Anolis* lizards, these atypical sex determining chromosomes are thought to have arisen from sex chromosome degeneration. This degeneration is hypothesized to be the product of the evolution of sex chromosomes from fusion with autosomes or fission of the X chromosome (Gamble et al. 2013). *Anolis sagrei* belongs to the group of reptiles with heteromorphic XXY sex chromosomes for males and XX sex chromosomes for females (Gamble et al. 2013).

When looking at resulting sex of an organism, some studies have shown that exposure to sex steroid hormones during embryonic development can have an effect (Wibbels et al. 1991, Bull et al. 1988, Freedberg et al. 2006). For instance, in the Spiny Softshell Turtle, *Apalone spinifera*, exposure to exogenous estradiol was seen to have a feminizing effect (Bull et al. 1988). *A. spinifera* is a species lacking heteromorphic sex chromosomes, or rather their sex chromosomes are homologous with each other. Since a species with homologous sex chromosomes could undergo a complete sex reversal, the

question arose if the same could be done in a species with heteromorphic chromosomes. Studies of avian and mammalian species that have heteromorphic chromosomes have resulted in evidence that estrogens cannot override male sex determination, however it has been seen to occur in a few reptilian cases (Freedberg et al. 2006).

There is a defining stage at which sex becomes irreversible for most species without ability to change sex (meaning not protogynous and protandrous fish). This stage in environmental sex determination has been defined as the so-called sensitive period. This is evidenced in a study by Wibbels et al. (1991). This study used the eggs of red-eared slider turtles (*Trachemys scripta*), a TSD species. The eggs were incubated at either male or female producing temperatures until they reached a certain stage of embryonic development (a stage between stage 14 and stage 22, which is about the middle third of incubation, depending on treatment group) and then were switched to the opposite sex producing temperature (Greenbaum and Carr 2001). It was noted that shifts in temperature at earlier stages changed the course for sex determination. The relative stage in development where sex became irreversible regardless of shift in incubation temperature was around embryonic stage 19-20. This is the period shortly after the gonadal ridge develops and the cortex and medullary regions begin to commit to one form of development for this species, or at about 50% of incubation duration (Wibbles et al. 1991).

Hormones of multiple origins (e.g., maternal, embryonic) may play important roles during development, shaping early development and subsequent phenotype. Yet, there is no clear mechanism or pathway known by which a developing embryo distinguishes between its own hormones and maternal hormones. Therefore, maternal

hormone exposure may influence offspring fitness (Uller et al. 2007). Support for the likelihood of maternal effects (or the change in offspring phenotype as a result of maternal influence) via steroids includes the fact that steroid hormones are lipophilic and can pass between yolk and embryo, and the eggs of many reptiles and closely related birds contain maternal steroid hormones that may differ extensively among clutches (Radder and Shine 2007; Andersson et al. 2004). Maternal steroid hormones have influenced sex determination and resulting fitness in some cases (Radder and Shine 2007). Maternal steroid levels can change in response to environmental conditions, which would result in a change in exposure of the developing embryo (Daisley et al. 2005; Schwabl 1996a,b). It has also been suggested that females are able to allocate different amounts of hormones to their eggs and therefore offspring in response to these changing environmental conditions, a necessity if the maternal effects are to be adaptive (Andersson et al. 2004).

Additionally, maternal steroid allocation can have differential effects on fitness of resulting offspring. Yolk T levels have had varied effects, both positive and negative, on offspring phenotypes depending on the species. In some cases, such as canaries (*Serinus canaria*) high T levels lead to increased growth rates along with increased begging behavior (Schwabl 1996, b). In other cases, such as with the American kestrel (*Falco sparverious*), high T levels lead to reduced growth and survival rate (Daisley et al. 2005). High T levels have even been found to decrease immune function of offspring (Andersson et al. 2004).

Many of the studies of maternal effects, both mentioned above and otherwise, have examined altricial species, or species requiring parental care. Less research has been

done on the level of maternal effects in precocial reptilian species such as anole lizards. It is known that for one anole species, *Anolis carolinensis*, sex differences in T levels are apparent, with resulting male embryos having a higher level of T both in the oviduct and at oviposition (Cohen and Wade 2010). This suggests there is some variance in maternal allocation of steroid hormones, though the allocation is possibly from passive influences due to the mother's circulating plasma hormones. These circulating plasma hormones have more influence on yolk hormone levels than the hormones acquired from the embryonic gonads during embryonic development for *A. carolinensis* (Cohen and Wade 2010).

3.2.2 Sex Steroid Hormones, Influencers of Sex Ratios and Physiology

Sex steroid hormones and their effects have been studied widely within various reptile models. Within these studies of reptiles, two sex steroid hormones, E_2 and T, have had varied effects of either feminization or masculinization of resulting offspring such as the feminization of spiny-softshell turtles from E_2 exposure (Bull et al. 1988). In *Anolis carolinensis*, the Green Anole, these hormones have been examined and found to be an important contributor to reproductive physiology with maternal contributions of these sex steroid hormones being present within the yolk of developing eggs. (Lovern and Wade 2001). Support for this occurrence of maternal hormones passing to the yolks of developing eggs is that sex steroid hormones are lipophilic and can pass through embryonic membranes during vitellogenesis (Radder and Shine 2007). Other studies have attributed sex ratio bias to a form of cryptic sex ratio bias, where large sires will produce more sons and small sires will produce more daughters. One such study investigating

Anolis sagrei lizards found that paternal sire condition shifted from a female biased sex ratio to a male biased sex ratio as paternal condition increased (Cox et al. 2010).

In this study, we chose to examine the effect that T and E_2 applied to the eggs had on the physiology of *Anolis sagrei* hatchlings as these are two hormones that are found in many sexually dimorphic reptiles and decrease when in the non-breeding season (Tokarz et al. 1998). *A. sagrei* is a species with heteromorphic chromosomes, and typically exhibits GSD (Gamble et al. 2013). This research addresses two primary questions. First, can exogenous exposure of sex steroid hormones to oviposited eggs affect sex and other related physiology of hatchling and adult *A. sagrei* in either a feminizing or masculinizing way? Second, if exposure occurs at two stages of embryonic development, is there a difference in sex steroid sensitivity? The two time points chosen would reflect maternal exposure to exogenous sex steroid hormones (as the yolk of developing eggs would contain solely maternally allocated hormones) and a point where sex steroid hormones should be present from developing gonads. If feminization occurs in the case of Estradiol (E_2) exposure, it would indicate that *Anolis* lizards with heteromorphic chromosomes have a similar mechanistic sex determination pathway to that of other reptiles.

3.3 Methods

Procedures described below were approved by the OSU IACUC (AS-17-8)

3.3.1 Anoles as a model species

The breeding season for *A. sagrei* follows a seasonal pattern beginning in March/April and ending in late August/September (Warner et al. 2009). Mating continues throughout the breeding season. Females lay single-egg clutches around every 10 days (Reedy et al. 2015). In general eggs are oviposited from alternating ovaries (Lovern and Passek 2002; Reedy et al. 2015). Females bury eggs in damp substrate, after which no further parental care is provided. Juveniles hatch from the eggs around 4-6 weeks after oviposition (Lovern et al. 2004a).

Anoline species are generally sexed through examination of the presence or absence of enlarged post anal scales. In males, two enlarged post anal scales can be observed immediately after hatching (Lovern et al. 2004a; Figure 6). This method however, may not accurately represent gonadal morphology in organisms treated with steroid hormones such as E_2 and T because exposure to exogenous steroid hormones does not necessarily fully affect gonadal structure or the development of the post anal scales, meaning if sex reversal occurs due to exogenous hormonal influence, it may not be a complete reversal or it may affect development of the gonads, but not the post anal scales (Xu et al. 2015).

3.3.2 Animal Husbandry and Processing

Seventy-five *A. sagrei* females were acquired from field-collected populations through a commercial supplier (South East Xotics, Palm Beach, FL) in 2018, and 48 females were acquired from field collected populations through a commercial supplier (Underground Reptiles, Deerfield Beach, FL) in 2019. Females were captured after the

breeding season had begun and were assumed to have already bred before arrival in the laboratory. Subjects were housed in 29-gallon group housing aquaria (3-5 females per tank). Tank environments were set up to mimic that of field conditions during the breeding season, filling the aquaria with moist peat moss and giving each tank at least two perches (dowel rods) along with at least one hide (concrete rocks). Additionally, females had access to water *ad libitum* and cages were sprayed with water daily. To simulate breeding season, the laboratory was kept on a 14:10 h light:dark schedule with a cage temperature range of 28-38℃ (hot and cool tank regions relative to the position of the basking light) during the day and 18℃ at night (Latham and Wade 2010). Lizards were fed a diet of mealworms, wax worms, and/or vitamin- and mineral-dusted crickets 3-4 times per week (Herptivite and MinerAll with D3, respectively; Lovern and Wade 2003).

3.3.3 Egg Collection

In reproductively active female anoles, a single-egg clutch is oviposited every 7-14 days from an alternate ovary with each successive oviposition (Lovern and Wade 2003). With females group housed, tanks were checked every day for the presence of a new egg so that eggs were collected within 24h of oviposition. If eggs were found, they were randomly assigned to a treatment group and processed prior to incubation.

3.3.4 Egg Processing and Treatment Application

A. sagrei eggs (N=338) were collected from female group housing aquaria and randomly assigned to different treatment groups. Upon collection, egg mass was

measured. To treat eggs, a solution of .5µg/µL of T or E_2 (total dose ~2.5µg) using a 95% ethanol vehicle was prepared based on similar experiments (Lance and Bogart 1994, Warner et al. 2017). This dosage was chosen based upon dosage amounts of similar experiments compared to the average mass of *A. sagrei* eggs (Lance and Bogart 1994, Warner et al. 2017). Eggs were treated at one of two time points day 0 (day of oviposition) or day 15 post oviposition, based on treatment assignment. Dosing was accomplished by adding the hormone solution directly to the outside of the egg using a 25µL Hamilton syringe (or just ethanol for controls). The two separate time points tested the effects of hormones on developing embryos before and after gonadal development. Once eggs were collected, they were placed into a jar containing a 1:1 mass:volume mixture of horticultural vermiculite and deionized water. Jars were then sealed with plastic wrap and a rubber band to retain moisture. Eggs were placed into an incubator averaging 28℃ for development for approximately 30 days (Lovern and Wade 2003).

3.3.5 Hatchling Processing Protocols

Upon hatching, each individual was sexed with the aid of a magnifying hand lens to look for the presence (male) or absence (female) of enlarged post anal scales (Figure 6). Once sexed, hatchlings were tagged using a traditional toe-clipping procedure giving each individual a unique ID (Figure 7). Hatchlings were then group housed in 10-gallon aquaria separated based on sex and treatment group until six months of age. Hatchlings were housed in a similar condition as females, in 10 gallon aquaria (this size was chosen as the hatchlings are much smaller compared to adults and it allowed for a smaller lid mesh to prevent escape) containing a hide, dowel rods for perching, and moist peat moss. Tanks were sprayed daily and hatchlings were fed a diet of vitamin- and mineral- dusted

(Herptivite and MinerAll with D3, respectively) pinhead crickets twice a week and non-dusted pinhead crickets twice a week. At six months of age, individuals were separated into individual 10-gallon aquaria and raised to reproductive maturity (approximately one year).

3.3.6 Adult Processing Protocols

At one year of age, individuals were measured for mass and snout-vent-length (SVL) before being euthanized. Euthanization was achieved by brief exposure to a lethal dose of inhaled isoflurane anesthesia followed by rapid decapitation. Upon decapitation, blood samples were collected from the trunk of 15 males and 28 females. Blood samples were then centrifuged and the plasma fraction was taken and kept at -70℃ until later radioimmunoassay (RIA) analysis (Lovern and Wade 2001). Immediately after blood samples were collected, the length of the primary dewlap cartilage was measured for each lizard to the nearest mm using digital calipers. Following that, necropsies were performed to examine the gonads of each lizard. Prior to euthanization, each individual was allowed a 10-minute intersexual interaction with a separate control conspecific, and then females checked for the presence of yolking follicles. Additionally, the left testis of each male was removed and weighed. As testes are on average of equal weight only one was needed. To check for sperm, males had their hemipenes everted and a sample taken for examination under a standard compound microscope at 100x magnification.

3.3.7 Steroid Assays

Radioimmunoassay (RIA) protocol adapted from the laboratories of John Wingfield (University of Washington) and Michael Moore (Arizona State University) by

the Lovern Laboratory at Oklahoma State University was followed. RIA procedures occurred over 4 days. Day one consisted of sample set-up. Plasma samples collected at the time of euthanization were thawed to room temperature and then vortexed. Plasma was drawn out of the tube using a Hamilton syringe and volume was recorded to the nearest microliter (μL) for each sample. The syringe was rinsed 2-3 times with ddH2O in between each sample.

After samples tubes were prepared, vortexed steroid standard (for each steroid being assayed) was added to each standard tube using a Hamilton syringe (aliquots of steroid standards were stored frozen in PBSg buffer). Standards were stored at concentrations of 500pg/100μL. For each standard tube, 10 μL of each steroid was used, which equaled 50 pg of T and 50 pg of E_2. After standards were added, we added 500 μL ddH2O to all tubes to provide sufficient volume from which to extract all samples and standards. To complete sample and standard tubes, a repeater pipet was used to dispense 20 μL of each tritiated steroid ("tracer") being assayed (~2000 dpm) into all samples and standard tubes excluding water blanks. Additionally, 20 μL was added to three 7 ml glass scintillation vials with 2 mL Ultima Gold scintillation cocktail (Packard) to be used after 3 days to determine sample recoveries following extraction. All sample tubes were then vortexed for 4-5 seconds and covered with aluminum foil and refrigerated overnight.

Day 2 consisted of extraction and column chromatography. Extraction of steroids from plasma was performed with 100% freshly opened diethyl ether (to avoid the formation of peroxides). Using a fume hood, 2 mL ether was added to each tube using a repeater pipet. Each sample was then vortexed for ~15 seconds and the ether phase (top) was transferred using a Pasteur pipette to clean, labeled 13x100 mm test tubes. A new

Pasteur pipette was used for each sample. This process was repeated once for each sample. The second ether fraction was transferred to the first ether extract test tube, and samples were then dried under nitrogen in a water bath at 37° C for ~15 minutes. Using a repeater pipette, 500 µL of 10% ethyl acetate:isooctane (EA:I) was added to each test tube and then vortexed, covered with parafilm, and set in the refrigerator for a minimum of 1 hr.

Column chromatography was then run on each sample tube. Columns were made using Fisher 5mL pipettes and 3-mm glass beads. Their purpose was to provide one additional step for the removal of neutral lipids as well as the individual isolation of steroids of interest; in this case, T and E_2. Short columns were run, therefore they were packed with 4 ml of the glycol phase and 1 ml of packed celite, of which the lower 0.5 mL was the water phase. Each column was filled with isooctane and blown through under high pressure with nitrogen. An angle rack was used to collect the isooctane into a glass collecting dish. Each sample was then vortexed for ~3-5 seconds and transferred to the column using a Pasteur pipette (using a new one for each sample). Samples were forced into the columns using nitrogen. With samples in columns, increasingly polar solvents were added and forced through to separate desired fractions into test tubes. Collected fractions (20% ethyl acetate in isooctane for T and 40% ethyl acetate in isooctane for E2) were then dried in 37° C water bath under nitrogen. Dried fractions were then resuspended into 500 µL PBSg at room temperature using a repeater pipette and vortexed for 4-5 seconds. They were then covered with parafilm and placed in the refrigerator overnight.

Day 3 consisted of RIA set-up. Reagents (PBSg, standard, tracer, antibody, and sample tubes) were allowed to reach room temperature. Each sample was vortexed for 4-5 seconds and then a 50-µL aliquot was placed into scintillation vials with 2 ml of Ultima Gold. Vials were capped and vortexed for 3-5 seconds, then 200-µL samples of each were transferred into two labeled 12x75-mm test tubes for the RIA. The standard curve was run in triplicate. Using a repeater pipette, 100 µL of antibody was placed into all samples (except total counts and non-specific binding) and each tube was then gently swirled. Following that, a repeater pipet was used to dispense 100 µL of tracer into all tubes. All tubes were then vortexed gently for 1-2 seconds, covered with aluminum foil, and placed in the refrigerator overnight.

Day 4 consisted of RIA completion. A centrifuge was run at 2200 rpm for 10 minutes to cool to 4°C. Dextran-coated charcoal in PBS (no gelatin) was added to standards and samples to stop the assay and remove unbound tracer. A 15 min timer was started and charcoal solution was rapidly added to all tubes. Sample racks were then vortexed manually for 10 seconds and tubes were placed in the centrifuge. After 15 min, the centrifuge was run at 2200 rpm for 10 min at 4°C. Supernatant was decanted into scintillation vials and 3.5 mL of Ultima Gold was added. Vials were then capped and vortexed. Count was then taken on scintillation counter to 2% error or 15 minutes. Counts were not run in duplicate as there was not enough blood plasma to run two sets of RIA analysis. Each assay was run singly, and the coefficient of variation for E_2 was 2.5% while the coefficient of variation for T was 55%.

3.3.8 Data Analyses

We calculated sex ratio as the number of female hatchlings divided by the total number of hatchlings. We investigated possible sex differences in sex and adult physiology by two experimental groups: Hormone Treatment and Treatment Day. To test if there was a difference in sex ratio we used Pearson Chi-squared test of heterogeneity to test the effects of Hormone Treatment and Treatment Day, on Sex separately as these groups are all categorical. We calculated the mean values of male offspring and female offspring separately when comparing adult physiology as *A. sagrei* is a sexually dimorphic species. A factorial analysis was used to test the interaction of our two experimental groups as well as each main effect for the following variables: SVL (Hatchling, Day 30, Day 60, and 1 Year), mass (Hatchling, Day 30, Day 60, and 1 Year), Plasma E_2 concentration, Plasma T concentration, Dewlap Cartilage Length, and Left-Testis Weight. SVL and mass were tested separately between time points due to significant changes in sample size between time-points due to natural deaths. If significance was found within our Two-way ANOVA for the Hormone Treatment, a Tukey *post-hoc* test was used to test which of the three hormones in Hormone Treatment was significantly different. Significance level was accepted at $p < 0.05$. Eggs were randomly assigned to the two treatment groups to prevent influence of maternal condition or size on the resulting physiology analysis. All data analyses were performed using IBM SPSS software.

3.4 Results

3.4.1 Sex Ratios and Egg Mortality

There was a significant relationship between E_2 and sex ratio. Sex was determined using post-anal scales and then compared to internal sex post-euthanization (sex results were the same in all instances). There was a significant association between the production of males and females and Hormone Treatment for both Day 0 groups and Day 15 groups pooled together (Pearson Chi-squared test of heterogeneity: $X^2 = 17.7063$, df = 2, $p < 0.001$; Figure 9). Eggs treated with E_2 were significantly more likely to result in female offspring. This bias of female production over male production for the Estradiol Treatment was seen only in eggs treated at Day 0 (X^2=16.254, df = 2, $p < 0.001$; Figure 10) and not in eggs treated at Day 15 ($X^2 = 3.593$, df = 2, $p > 0.05$ igure 11). This bias of female production over male production was not due to a difference in the mortalilty rate of different hormone treatments. Hormone Treatment did not have a significant effect on the rate of egg-death (Pearson Chi-squared test of Heterogeneity: $\chi^2 = 1.170$, df = 2 , $p > 0.05$).

3.4.2 Blood Plasma Hormone Levels

Neither Hormone Treatment nor Treatment Day had a significant effect on the level of Plasma T in males (Two-way ANOVA: Hormone Treatment, $F_{2,18} = 0.081$, $p>0.05$; Treatment Day, $F_{1,18} = 0.589$, $p> 0.05$). The interaction of Hormone Treatment and Treatment Day was also found to be insignificant ($F_{2,18} = 0.671$, $p> 0.05$) (Figure 12). In addition, neither Hormone Treatment nor Treatment Day had a significant effect on the level of Plasma E_2 in males (Two-way ANOVA: Hormone Treatment, $F_{2,18} = 0.214$, $p>0.05$; Treatment Day $F_{1,18} = 0.840$, $p> 0.05$). The interaction of Hormone Treatment and Treatment Day on Plasma E_2 concentration was also found to be insignificant ($F_{2,18}$ =

0.069, $p > 0.05$) (Figure 13). Simple main effects analysis showed that Plasma T conentration in females was not significantly affected by Hormone Treatment or Treatment Day (Two-way ANOVA: Hormone Treatment, $F_{2,36} = 1.633$, $p > 0.05$; Treatment Day $F_{1,36} = 0.474$, $p > 0.05$). The interaction of Hormone Treatment and Treatment Day on Plasma E_2 concentration was also found to be insignificant ($F_{2,36} = 1.069$, $p > 0.05$) (Figure 14). Plasma E_2 concentration in female adults was not found to be significantly affected by Hormone Treatment or Treatment Day (Two-way ANOVA: Hormone Treatment, $F_{2,36} = 2.484$, $p > 0.05$; Treatment Day $F_{1,36} = 0.860$, $p > 0.05$). We did not find that the interaction of Hormone Treatment and Treatment Day significantly affected plasma E_2 concentration in females either ($F_{2,36} = 2.484$, $p > 0.05$). However, conclusions from these results must be drawn with caution due to low sample size of each subgroup.

3.4.3 SVL, Mass, and Dewlap Cartilage

Female Hatchling mass and SVL were not significantly affected by either Hormone Treatment or Treatment Day at any of the time-points tested: Hatchling (Hatch Day), 30 Days, 60 Days, and 1 Year post-hatch (Table 1). Hormone Treatment and Treatment day did not significanlty affect Male SVL (Two-way ANOVA: Hormone Treatment $F_{2,83} = 0.133$, $p > 0.05$; Treatment Day $F_{1,83} = 0.200$, $p > 0.05$). The interaction of Hormone Treatment and Treatment day on Male Hatchling SVL however, was significant ($F_{2,83} = 4.684$, $p = 0.012$)(Figure #15). Hatchling males treated with E_2 on Day 15 Post-Oviposition were longer than controls, whereas males treated with E_2 on Day 0 Post-Oviposition were shorter than controls. In addition, Treatment Day

significantly affected Male Hatchling mass (Two-way ANOVA: $F_{1,83} = 4.865$, $p = 0.030$), but Treatment Hormone did not ($F_{2,83} = 1.156$, $p > 0.05$)(Figure 16). The interaction of Hormone Treatment with Treatment Day also was not significant ($F_{2,83} = 1.386$, $p > 0.05$). Hormone Treatment significantly affected the SVL of males at 30 Days post-hatch (Two-way ANOVA: $F_{2,37} = 4.54$, $p = 0.017$), but Treatment Day did not ($F_{1,37} = 1.094$, $p > 0.05$)(Figure 17). The interaction of Hormone Treatment with Treatment Day also was not significant ($F_{2,37} = 1.229$, $p > 0.05$). In post-hoc comparisons, males treated with both Estradiol and Testosterone had significantly smaller SVLs than controls (Estradiol $p = .038$; Testosterone $p = .043$). Additionally, Hormone Treatment signifcantly affected the mass of males at 30 Days post-hatch (Two-way ANOVA: $F_{2,37} = 7.447$, $p = 0.002$), but Treatment Day did not ($F_{1,37} = 2.461$, $p > 0.05$)(Figure 18). The interaction of Hormone Treatment and Treatment Day also was not significant ($F_{2,37} = 2.461$, $p > 0.05$). In post-hoc comparisons, males treated with both Estradiol and Testosterone had significantly lower mass compared to controls (Estradiol $p = 0.004$; Testosterone $p = 0.018$). Male SVL and Mass at 60 Days and 1 year post-hatch were not significantly affected by Hormone Treatment or Treatment Day (Table 2).

Neither Hormone Treatment nor Treatment Day significantly affected the length of female dewlaps (Two-way ANOVA: Hormone Treatment, $F_{2,29} = 2.105$, $p > 0.05$; Treatment Day, $F_{1,29} = 0.007$, $p > 0.05$). The interaction effect between Hormone Treatment and Treatment Day on length of dewlap cartilage of female anoles also was not significant ($F_{2,29} = 0.911$, $p > 0.05$) (Figure 20). Treatment Hormone significantly affected Dewlap Length in males (Two-way ANOVA: $F_{2,23} = 3.779$, $p = 0.038$), but Treatment Day did not ($F_{1,23} = 0.014$, $p > 0.05$). The interaction of Hormone Treatment

with Treatment Day also was not significant ($F_{2,23} = 1.044$, $p > 0.05$). In post-hoc comparisons, males treated with Estradiol had significantly smaller Dewlap Lengths compared to controls ($p = 0.027$), but Dewlap Length of males treated with Testosterone compared to controls was not significantly different ($p > 0.05$) (Figure 19).

3.4.4 Reproductive Physiology

Post-euthanization, individuals were necropsied to check for the presence of yolking follicles in females, and to weigh the left testis of males. Females of all treatment groups were more likely to have yolking follicles compared to the controls (Pearson Chi-squared test of heterogeneity: $\chi^2 = 14.583$, df = 2, $p < 0.001$). Hormone Treatment and Treatment Day did not have a significant effect on male left testis weight (Two-way ANOVA: Hormone Treatment, $F_{2,23} = 1.924$, $p > 0.05$; Treatment Day, $F_{1,23} = 2.707$, $p > 0.05$). The interaction of Hormone Treatment and Treatment Day was also found to be insignificant ($F_{1,23} = 0.019$, $p > 0.05$). Post Hoc tests however, revealed that males treated with T on Day 0 post-oviposition had a significantly lower left testis weight than controls ($p = 0.019$) (Figure 21). This significant result of a lower left testis weight for males treated with T on Day 0 post-oviposition could have been obfuscated in the main effects tests due to the low sample size of Day15 post-oviposition.

3.5 Discussion

3.5.1 Sex Reversal in Anolis sagrei lizards

In this study we examined the relationship of T and E_2 on the sex determination pathway of *A. sagrei* by investigating two time points for hormone application. The exact pathway of maternal hormone transference to developing eggs is still unknown. However, maternal alterations of yolk hormones can influence postnatal gene expression and the passage of androgens from mother to embryo are flexible (Lutyk et al. 2017; Okuliarova et al. 2014). Once these hormones have been transferred to offspring in oviparous species, it is metabolized by the embryo and these embryos have been hypothesized to have active control over yolk steroid use (Kumar et al. 2018). Typically, in *A. sagrei*, a lizard with heterogametic sex determination, sex ratio of resulting offspring tends towards a 50:50 ratio of male to female (Cox and Calsbeek 2010, Walguarnery 2007). Our original hypothesis was that exposure to E_2 or T would have an effect on the physiology of resultant offspring in either a masculinizing or feminizing way. Our results suggest that exposure to E_2 at a time point that could represent maternal origin, leads to a female sex ratio bias and the sex of resultant offspring is set by day 15 of incubation. Incubation day 15 is the embryonic developmental stage in *A. sagrei* in which developing gonads begin producing their own versions of sex steroid hormones. We have observed similar effects of estradiol exposure in species with GSD resulting in a female sex ratio bias.

One such example is in a study of female zebra finches, *Taeniopygia guttata*, injected E_2 that produced significantly more female than male offspring (von Engelhardt et al. 2004). The difference between that study and our own however, was that the sex ratio affected by E_2 exposure was the secondary sex ratio, whereas in our own study, the

primary sex ratio was affected. Another example of a female sex ratio bias after exposure to E_2 is in a study on the Indian skipper frog, *Euphlyctis cyanophlyctis*, another species with GSD (Phuge and Gramapurohit 2015). Though these two examples are of non-reptiles, many studies of reptiles that exhibit TSD, have also seen this hormonal override of the resulting sex ratio being biased towards females (Crews et al. 1996; Jandegian et al. 2015). These examples point to E_2 being critical in the female sex determination pathway, and may be sufficient as a mediator in place of temperature or gene cues in the sex determination pathway.

3.5.3 Hormone Mediated Changes in Anolis sagrei Physiology

In addition to investigating the effects that E_2 and T had on the sex ratio of *A. sagrei* offspring, we examined how these sex steroid hormones affected their size and adult physiology. Plasma sex steroid hormone levels exhibit variances that are influenced by several factors: season, presence of conspecifics, and sex (Neal and Wade 2007). With this in mind, we wanted to test if maternal or exogenous increases in sex steroid hormones at an embryological development point had an effect on plasma hormone levels of adults. We found that the hormone treatments received as eggs did not cause a significant difference in adult plasma T or E_2 for either males or females. This suggests that adult hormone levels are not necessarily linked to embryological variance in hormone levels.

We also examined the effects of these two hormones on the growth of males and females at four different time-points: hatch day, 30-days post-hatch, 60-days post-hatch, and 1-year post-hatch. We found that female mass and SVL were not significantly affected by hormone treatment or treatment day. Male hatchling SVL was significantly

affected by the interaction of treatment day and hormone treatment. We found that males treated on Day 0 post-oviposition with E_2 were significantly shorter than controls, while males treated on Day 15 post-oviposition were significantly longer. In addition, males treated with both E_2 and T had significantly smaller SVL and mass at day 30 post-hatch compared to controls.

The differences in the Day 0 group treated with E_2 are similar to effects seen in a study with *Alligator mississippiensis*, the American Alligator, where E_2 treated eggs had a slower growth rate shortly after hatch that expedited later after hatching (Cruze et al. 2015). Based on this similarity, we can infer that in our study for males treated at Day 0, E_2 inhibited growth. To explain why males treated at Day 15 post oviposition had longer hatchling SVL than controls, we can look at a study of the snapping turtle, *Chelydra serpentine*. This study revealed that the yolk steroid hormones of these reptiles had declined by about 50% by the end of the sex differentiating period (Elf et al. 2002). In our study, the refreshed level of available sex steroid hormones potentially led to a change in size not capable for the group treated earlier.

In addition to these differences in body size, we found that males treated with E_2 had significantly smaller dewlaps than controls or T treated males. T has been shown to correlate with dewlap size in *A. carolinensis*, however, less research has been completed on these correlations in *A. sagrei* (Husak et al. 2007). Because T correlates with larger dewlap size it is possible that high levels of E_2 could inhibit T receptivity in males leading to a smaller dewlap size.

When investigating the effects of T and E_2 on the reproductive physiology of *A. sagrei,* we found that males treated with T on Day 0 post-oviposition had significantly

smaller left testis weights when compared to controls. As has been seen in garter snakes, T could result in a lower body size, or in this case testis size, by acting in an inhibitory manner (Crews et al. 1985). These results indicate that maternal allocation of sex steroid hormones, or conversely early embryonic exposure, could have detrimental effects on the adult life of resultant male offspring. Males that are on average smaller in size, tend to have smaller territories and more difficulty defending their territory (Bush et al. 2016). It would be worth investigating how the decreased size of treated males affects early survival rates in the wild.

3.5.3 Future Implications

These findings could have implications on future studies as they indicate that in the Cuban Brown Anole, maternal hormone allocations of estradiol to yolking follicles could have an effect on resultant offspring. Alternatively, there is the implication that eggs, when laid near water sources that have increased levels of estrogens or estrogen like pollutants could lead to a female bias that is not due to differential egg mortality. Such a phenomenon has been documented with turtles, such as a study with *Chrysemys picta*, which noted that exposure to E_2 and Bisphenol A (BPA) which is an estrogenic mimic chemical, led to disruption in sexual differentiation (Jandegian et al. 2015). With the prevalence of endocrine disrupting chemicals in aquatic environments some studies have noted that even if gonadal sex isn't disrupted, resulting offspring of exposed individuals may pass down compromised genetic fitness (Bhandari et al. 2014).

A. sagrei is a species that reproduces near these aquatic environments. It is also a generalist organism that has infiltrated urban environments including areas near water

treatment plants, which falls under those aquatic areas that contain endocrine disrupting chemicals (Tiatragul et al. 2017, Bhandari et al. 2014). *A. sagrei* is an increasingly invasive species that competes with the native green anole, *Anolis carolinensis*. With *A. sagrei* being an invasive species, having a higher female-biased sex ratio could increase its invasion success, as females can have multiple mates and store sperm for long periods to use at will. Future studies should examine more about when exactly gonadal hormones of *A. sagrei* begin to have an effect on sexual differentiation, as well as the effects of contaminated soil on the sex ratio of these lizards.

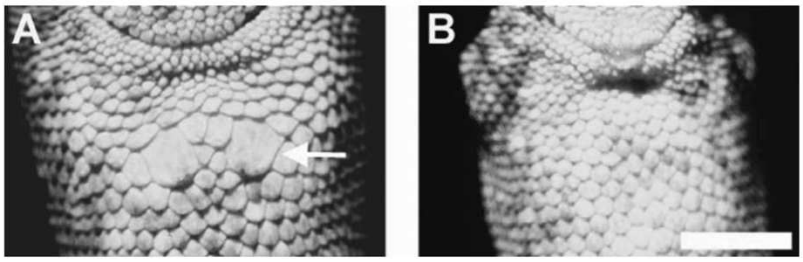

Figure 6: A: arrow indicates two post-anal scales in a male *Anolis carolinensis*. B: absence of post-anal scales in a female *A. carolinensis*. Taken from Lovern et al. 2004

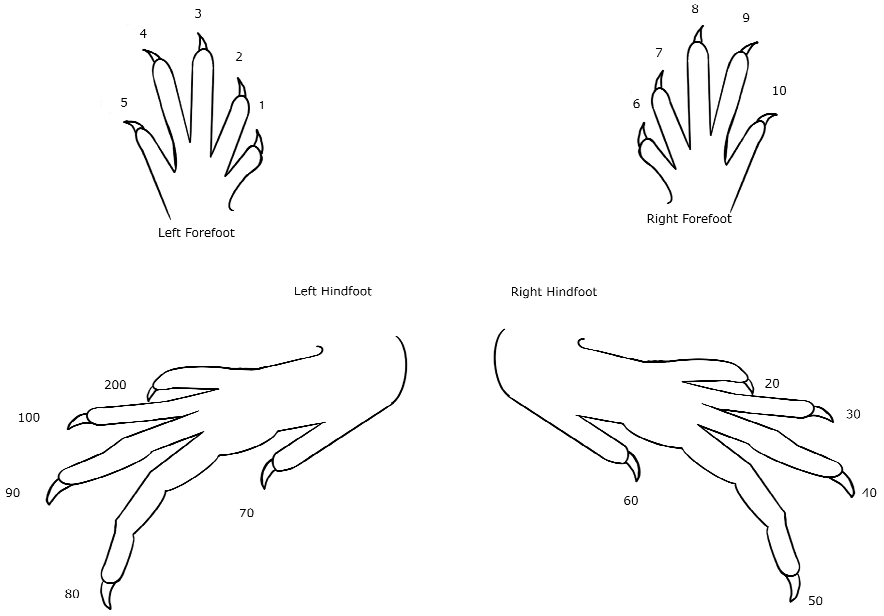

Figure 7: Diagram for unique ID toe clipping procedure. The corresponding ID number for each lizard is the sequence of clipped toes.

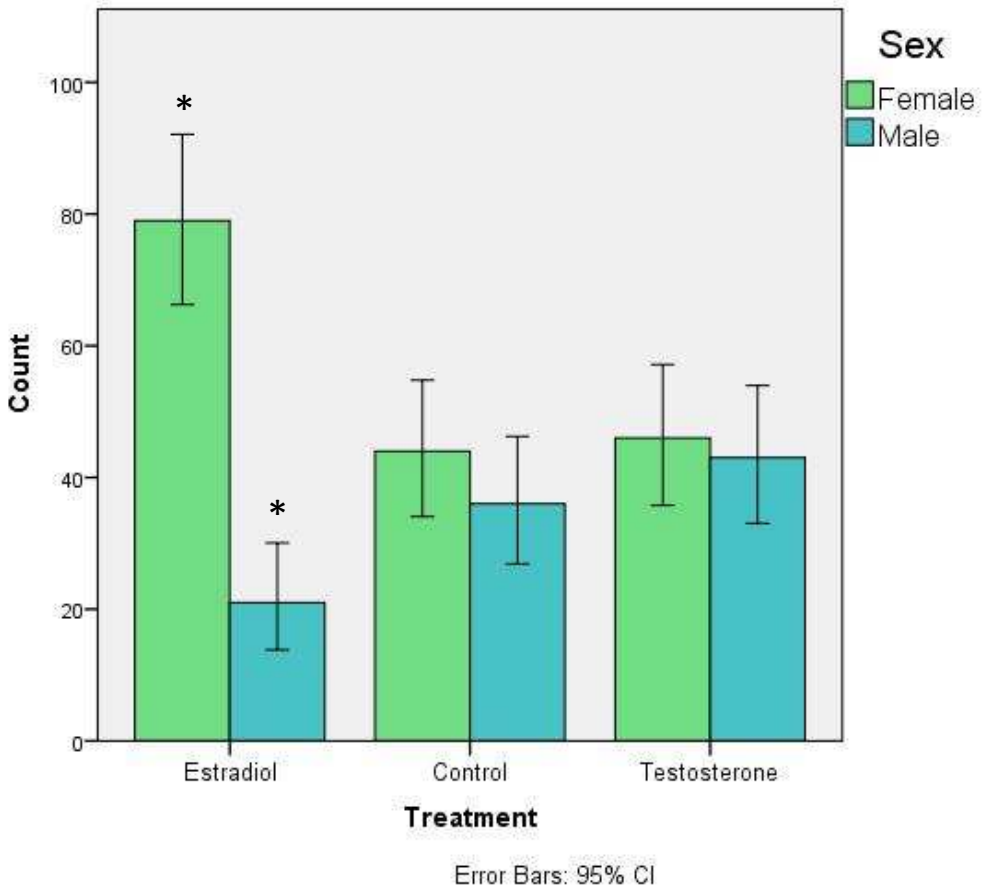

Figure 8: Number of males and females produced from *Anolis sagrei* eggs treated on Day 0 and Day 15 post-oviposition (pooled together) with Estradiol, Testosterone, or Control (Ethanol). An asterisk indicates significant difference ($p < 0.05$).

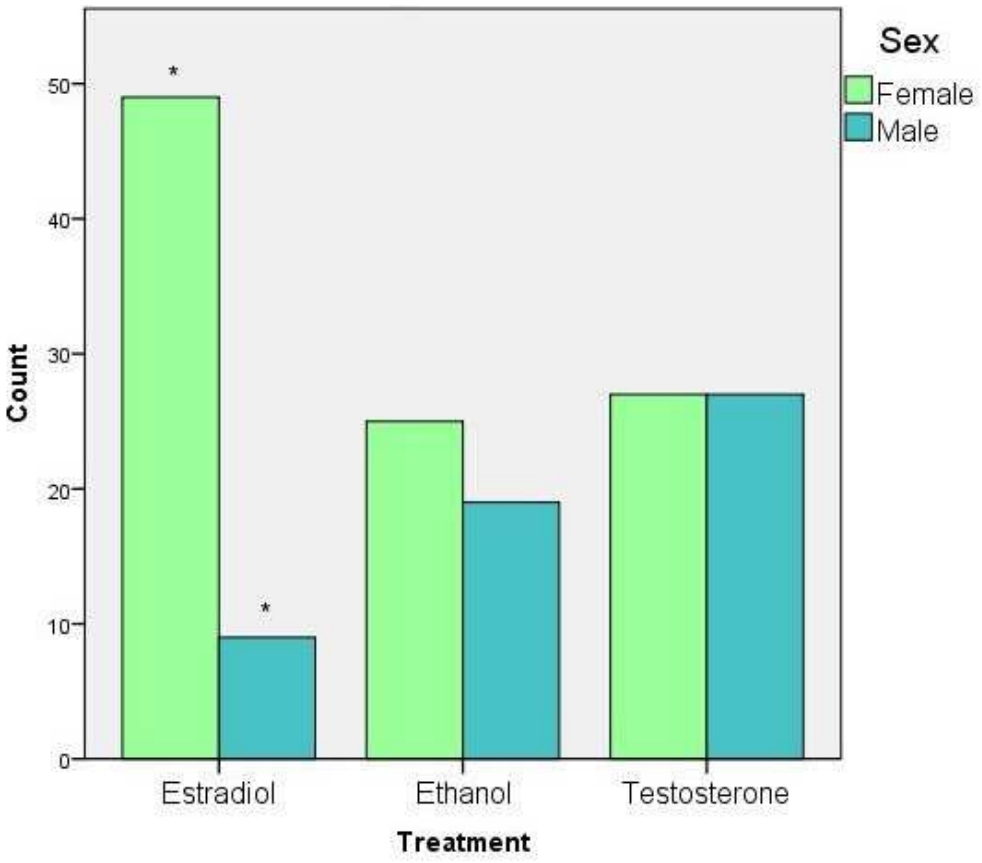

Figure 9: Number of males and females produced from *Anolis sagrei* eggs treated on day 0 post-oviposition with Estradiol, Testosterone, and control (ethanol). An asterisk indicates a significant difference ($p < 0.05$).

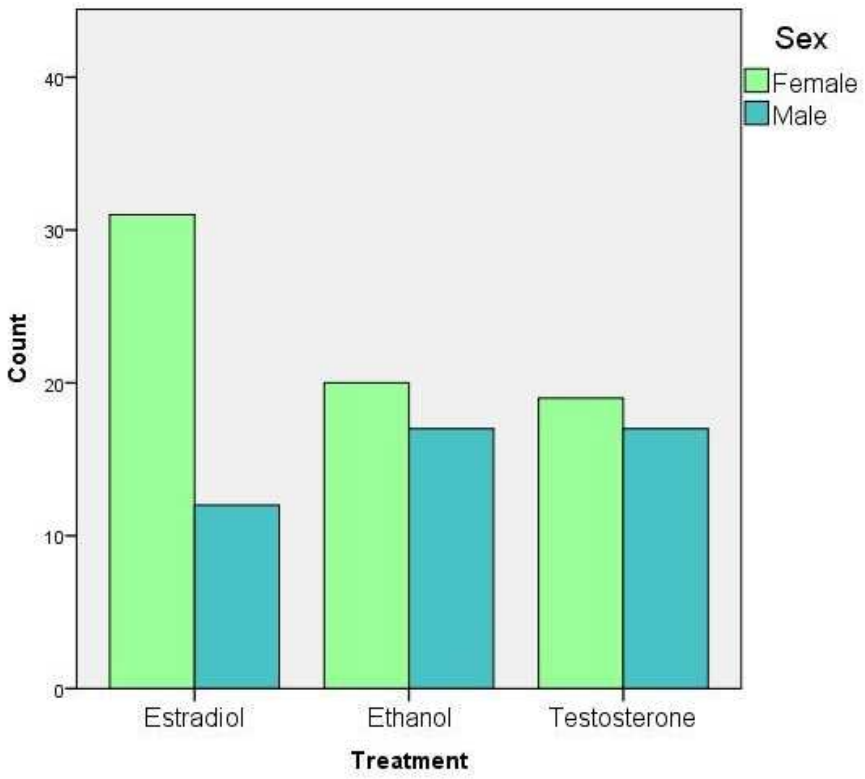

Figure 10: Number of males and females produced from *Anolis sagrei* eggs treated on day 15 post-oviposition with Estradiol, Testosterone, and control (ethanol).

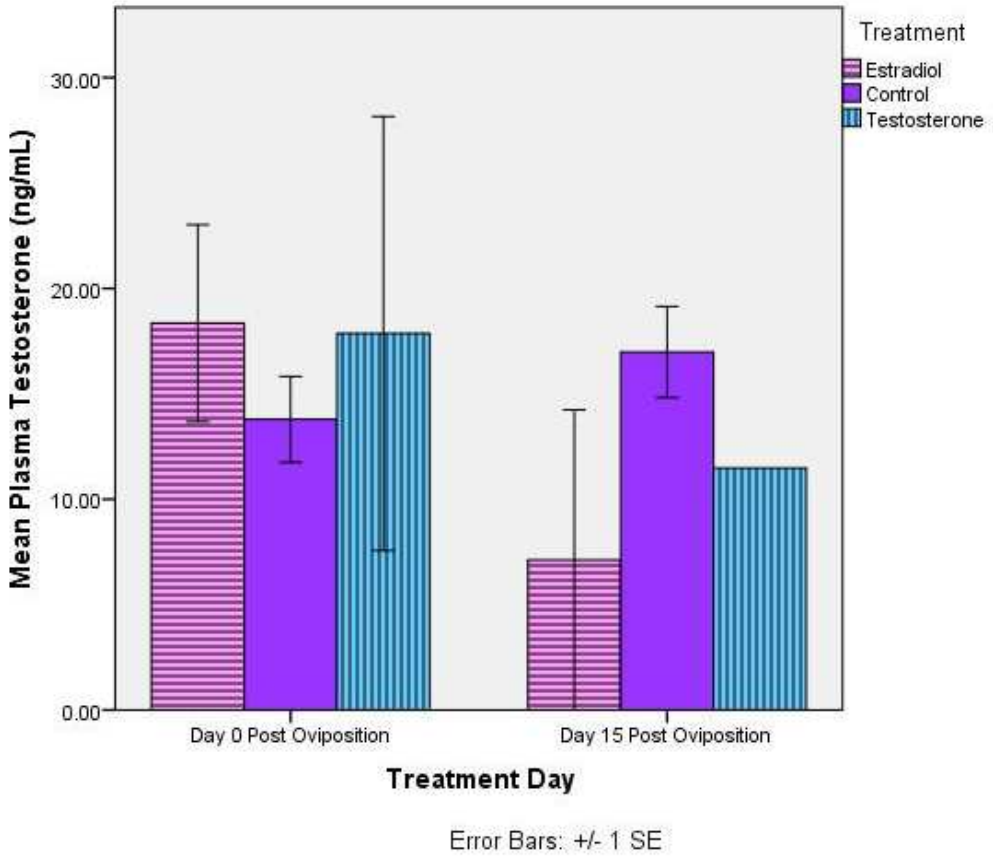

Figure 11: Mean (± SE) plasma testosterone (T) concentrations for adult *Anolis sagrei* males by Hormone Treatment and Treatment Day.

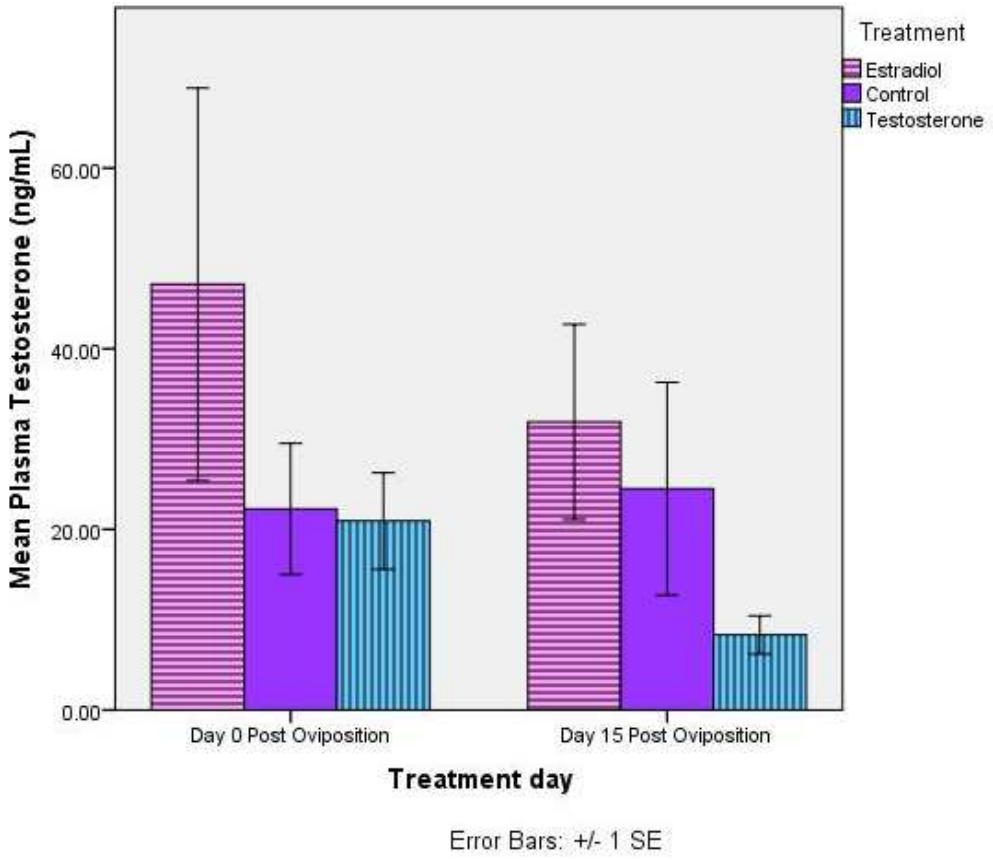

Figure 12: Mean (± SE) plasma testosterone (T) concentrations for adult *Anolis sagrei* females by Hormone Treatment and Treatment Day.

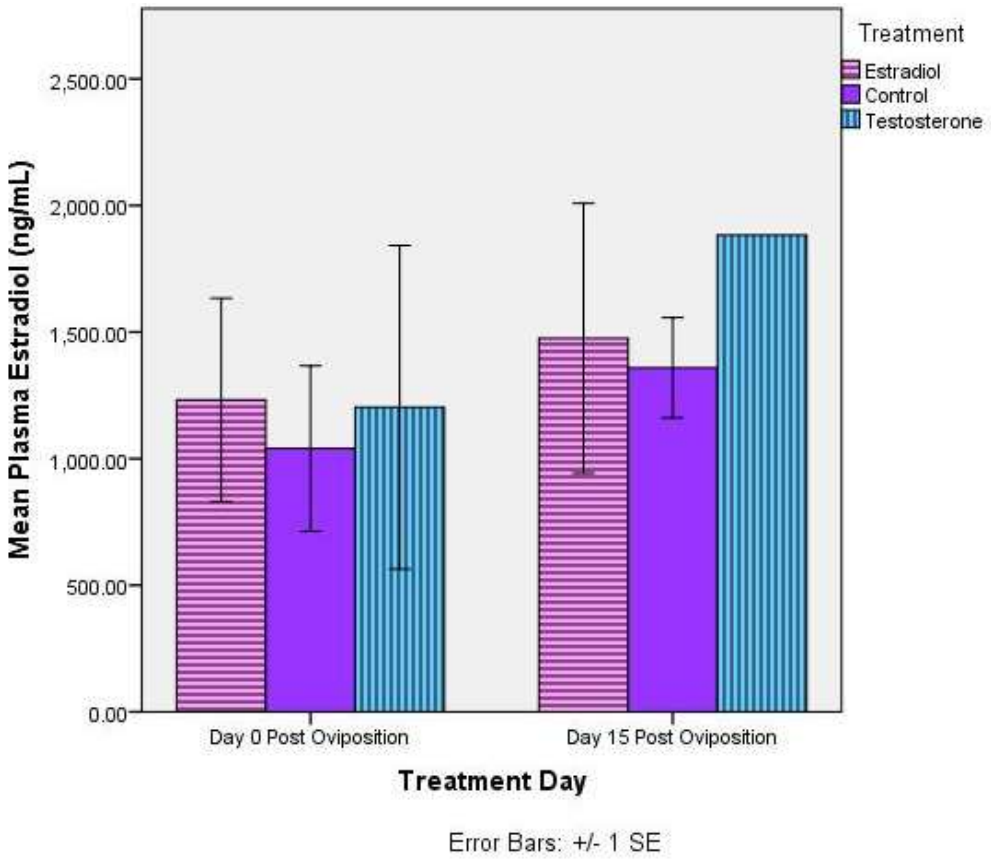

Figure 13: Mean (± SE) plasma estradiol (E_2) concentrations for adult *Anolis sagrei* males by Hormone Treatment and Treatment Day.

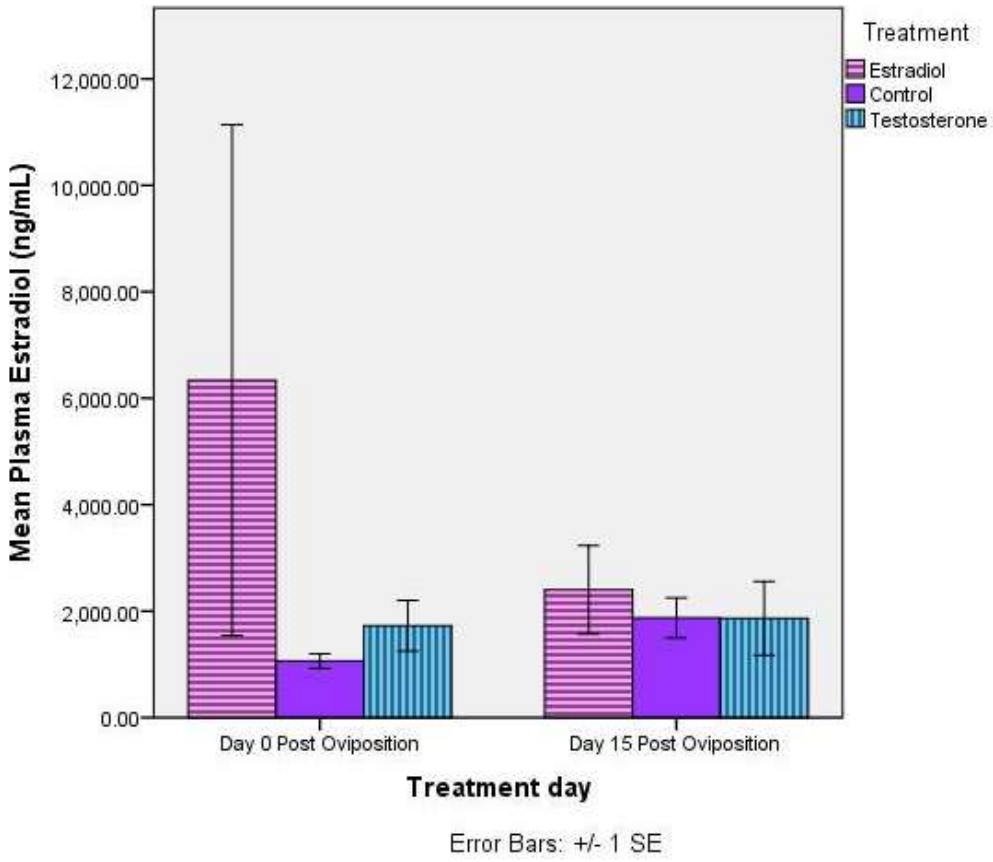

Figure 14: Mean (± SE) plasma estradiol (E_2) concentrations for adult *Anolis sagrei* females by Hormone Treatment and Treatment Day.

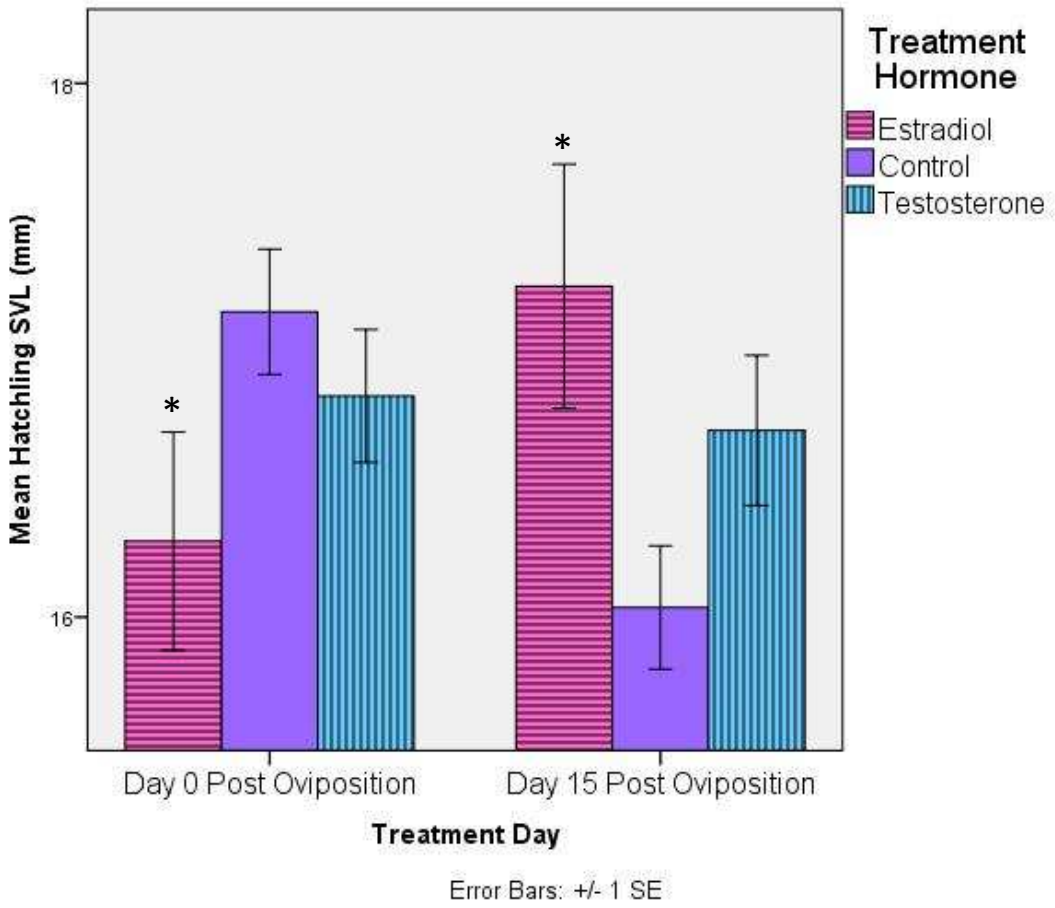

Figure 15: Mean (± SE) snout-vent-length (SVL) for male *Anolis sagrei* hatchlings measured day of hatching by Treatment Hormone and Treatment Day. An asterisk indicates a significant difference ($p < 0.05$).

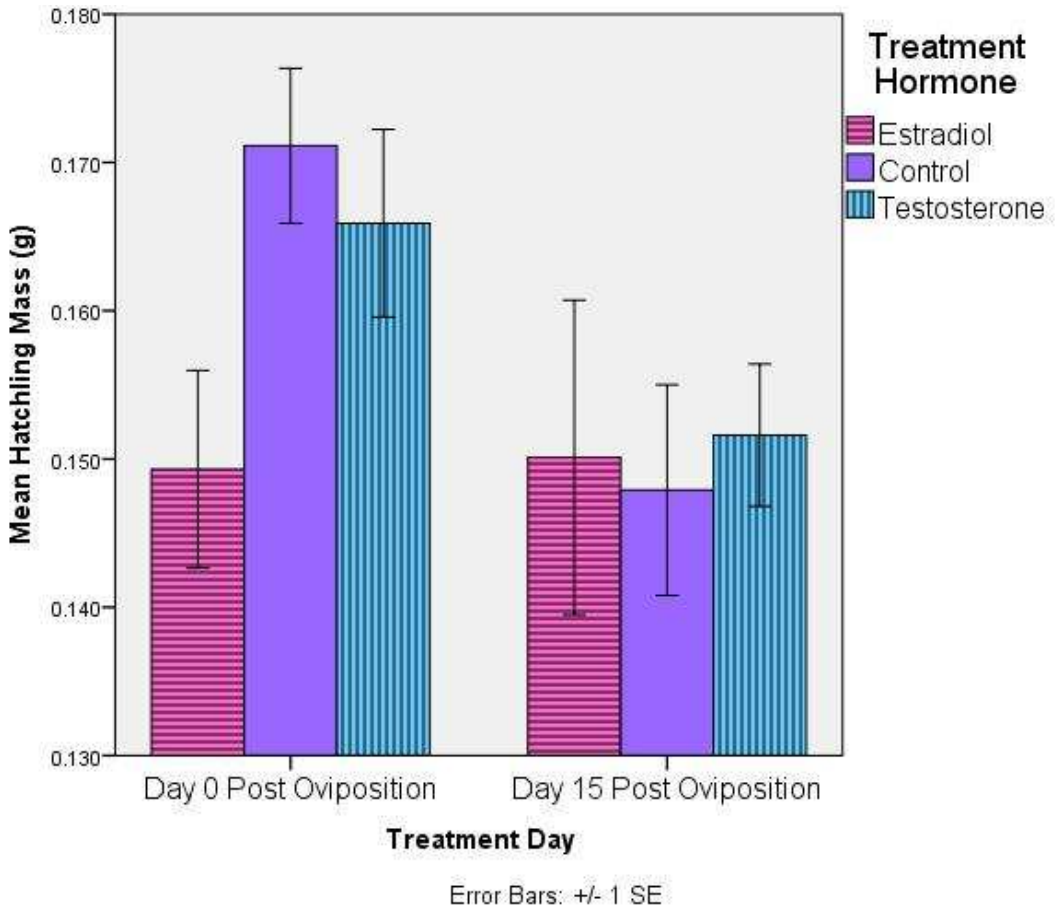

Figure 16: Mean (± SE) mass for male *Anolis sagrei* hatchlings measured day of hatching by Treatment Hormone and Treatment Day.

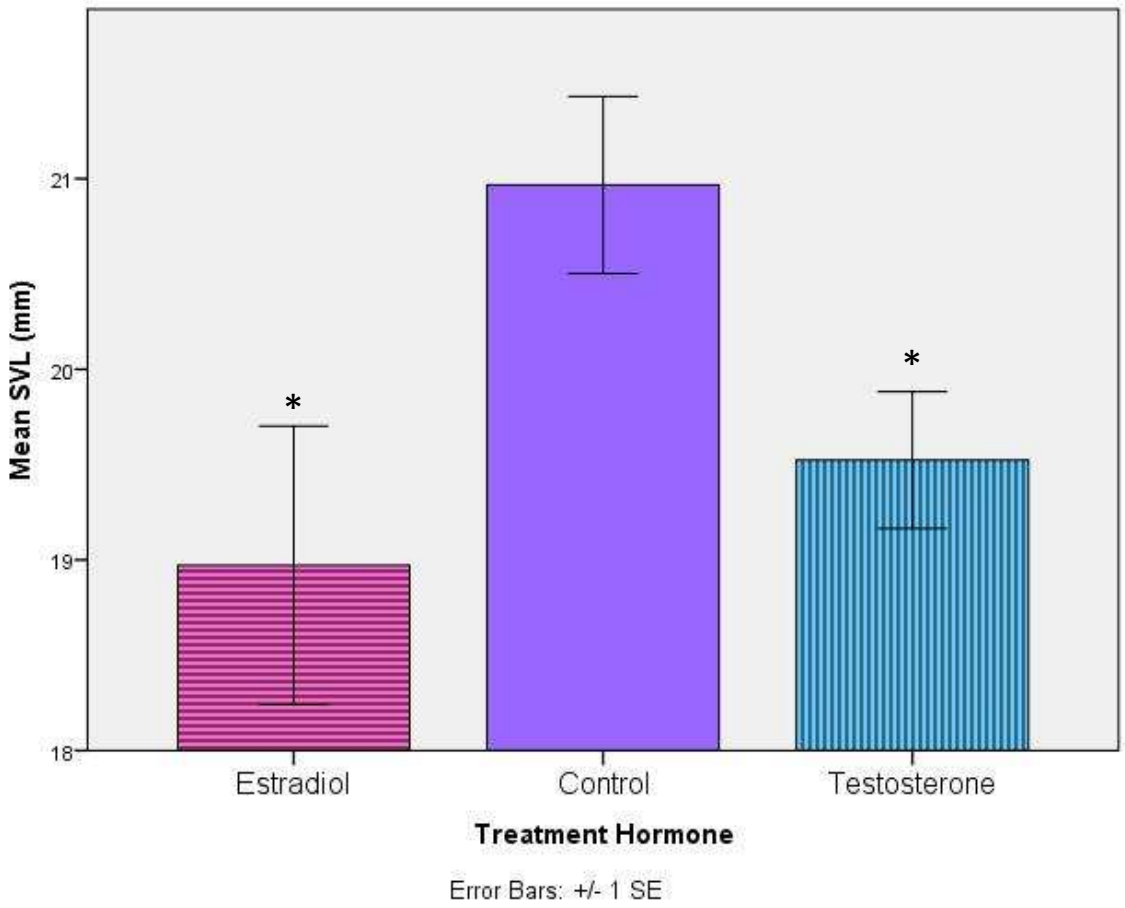

Figure 17: Mean (±SE) snout-vent-length (SVL) for male *Anolis sagrei* hatchlings measured on day 30 post-hatch by Treatment Hormone. An asterisk indicates significant difference ($p < 0.05$).

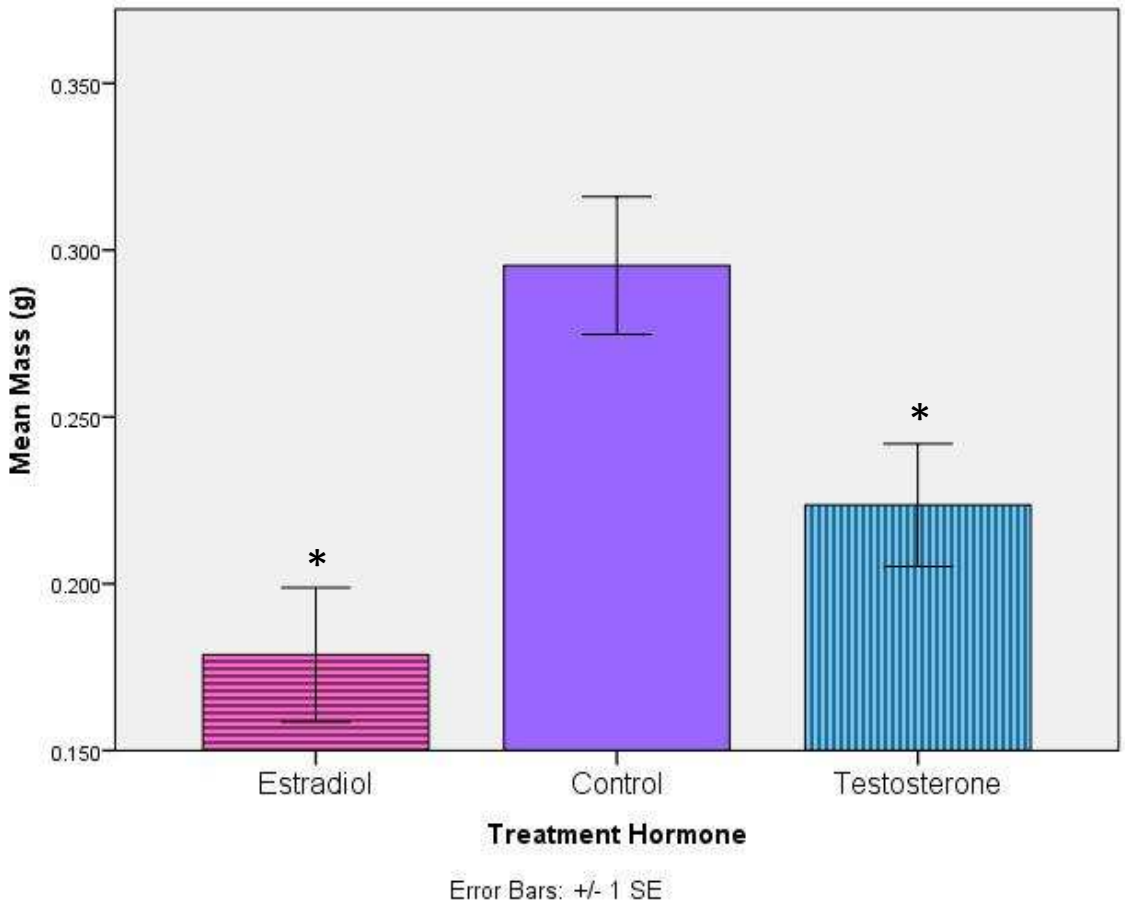

Figure 18: Mean (±SE) mass for male *Anolis sagrei* hatchlings measured on day 30 post-hatch by Treatment Hormone. An asterisk indicates significant difference ($p < 0.05$).

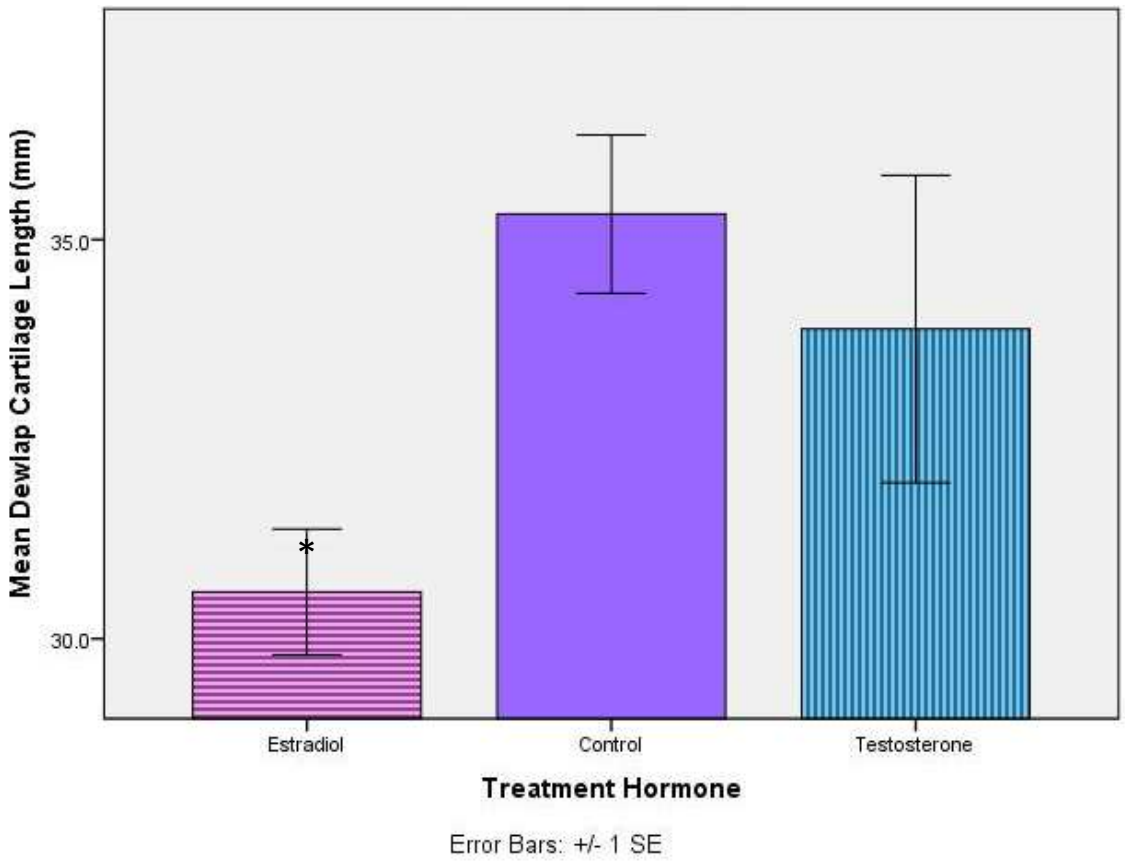

Figure 19: Mean (± SE) dewlap cartilage length for adult *Anolis sagrei* males by Treatment Hormone. An asterisk indicates a significant difference ($p < 0.05$).

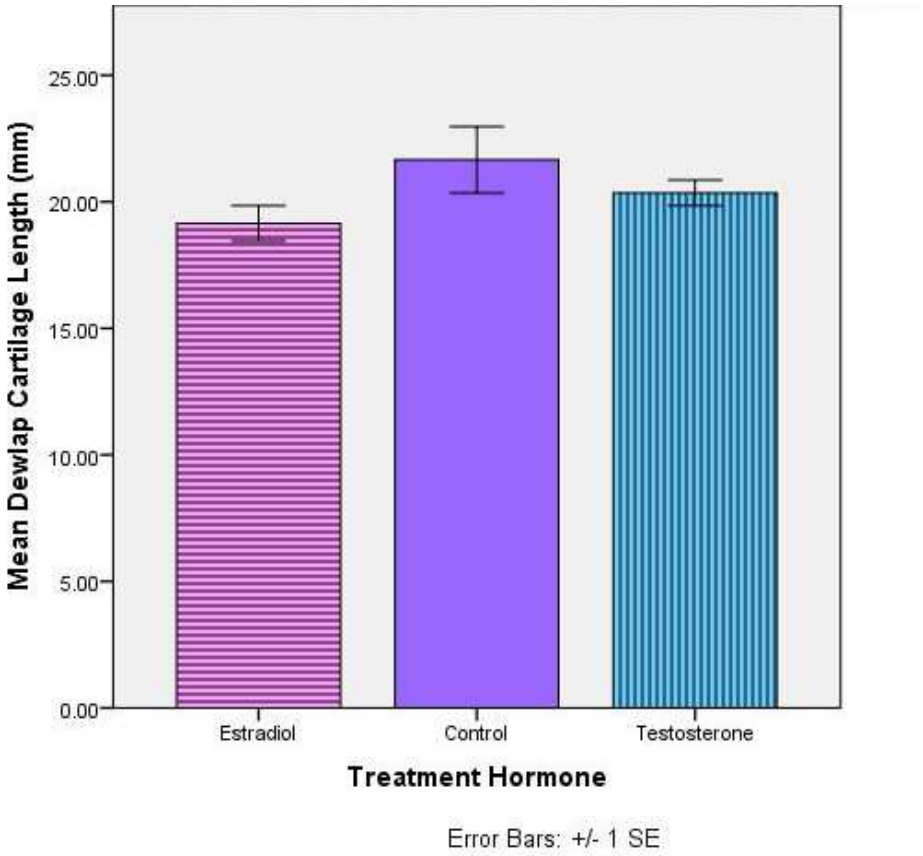

Figure 20: Mean (± SE) dewlap cartilage length for adult *Anolis sagrei* females by Treatment Hormone.

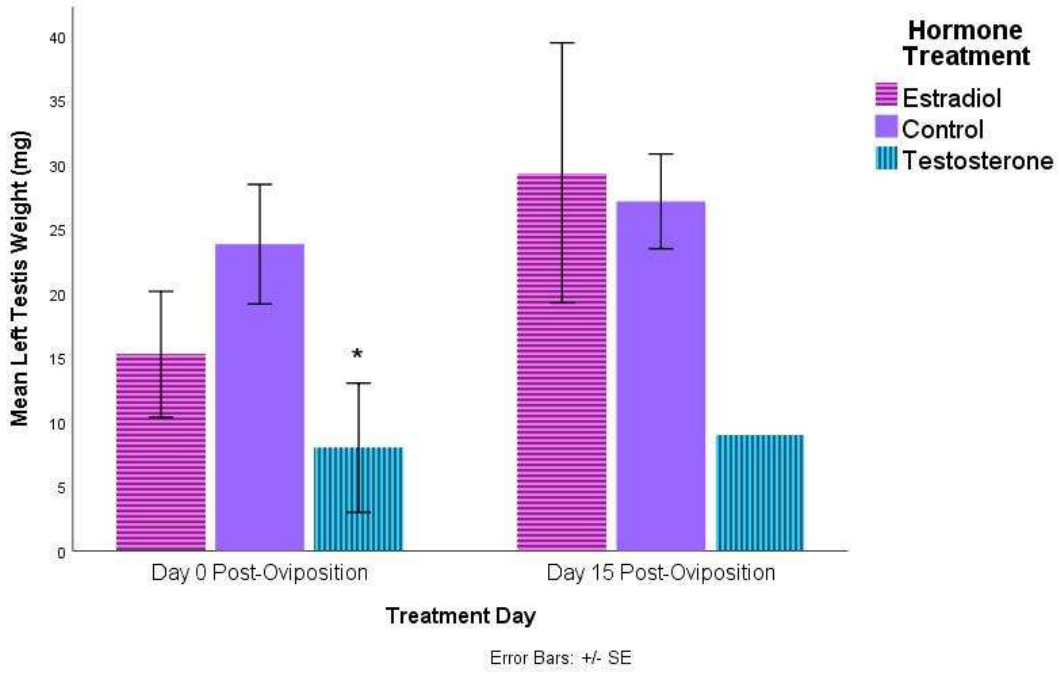

Figure 21: Mean left testis weight of *Anolis sagrei* males treated at one of two time points with one of three treatments: estradiol, testosterone, or control (ethanol). An Asterisk indicates significant difference($p < 0.05$).

Two-way ANOVA results measuring snout-vent-length (SVL) for *Anolis sagrei* Lizards

Parameter	Factor	SS	df	MS	F	*p*
Female Hatchling SVL (mm)	Hormone Treatment	3.561	2	1.780	1.157	3.170
	Treatment Day	5.752	1	5.752	3.783	0.055
	Hormone Treatment x Treatment Day	0.067	2	0.034	0.022	0.978
	Error	221.597	144	1.539		
Female 30 Day SVL (mm)	Hormone Treatment	0.116	2	0.058	0.019	0.981
	Treatment Day	2.499	1	2.499	0.830	0.365
	Hormone Treatment x Treatment Day	3.992	2	1.996	0.663	0.518
	Error	264.940	88	3.011		
Female 60 Day SVL (mm)	Hormone Treatment	17.187	2	8.593	1.550	0.160
	Treatment Day	11.218	1	11.218	2.024	0.221
	Hormone Treatment x Treatment Day	5.177	2	2.588	0.467	0.629
	Error	304.836	55	5.542		
Female 1 Year SVL (mm)	Hormone Treatment	0.438	2	0.438	0.091	0.913
	Treatment Day	2.298	1	1.149	0.035	0.853
	Hormone Treatment x Treatment Day	12.140	2	6.070	0.483	0.622
	Error	339.528	27	12.575		
Male Hatchling SVL (mm)	Hormone Treatment	0.182	2	0.182	0.133	0.717
	Treatment Day	0.550	1	0.275	0.200	0.819
	Hormone Treatment x Treatment Day	12.883	2	6.442	4.684	0.012*
	Error	114.135	83	1.375		
Male 30 Day SVL (mm)	Hormone Treatment	26.317	2	13.159	4.540	0.017*
	Treatment Day	3.170	1	3.170	1.094	0.302
	Hormone Treatment x Treatment Day	7.122	2	3.561	1.229	0.304
	Error	107.293	37	2.898		
Male 60 Day SVL (mm)	Hormone Treatment	23.894	2	11.947	1.781	0.198
	Treatment Day	10.535	1	10.535	1.570	0.227
	Hormone Treatment x Treatment Day	8.761	2	4.380	0.653	0.533
	Error	114.034	17	6.708		
Male 1 Year SVL (mm)	Hormone Treatment	172.193	2	86.097	2.925	0.076
	Treatment Day	65.448	1	65.448	0.331	0.571
	Hormone Treatment x Treatment Day	173.996	2	86.998	0.651	0.532
	Error	921.960	21	43.903		

Table 1: Two-way ANOVA results measuring snout-vent-length (SVL) for *Anolis sagrei* lizards treated with one of three Hormone Treatments: Estradiol, Testosterone, or Control (Ethanol). Hormone Treatments were applied at one of two different Treatment Days: Day 0 Post-Oviposition or Day 15 Post-Oviposition. An asterisk indicates a significant difference ($p < 0.05$).

Two-way ANOVA results measuring mass for *Anolis sagrei* Lizards

Parameter	Factor	SS	df	MS	F	*p*
Female Hatchling mass (g)	Hormone Treatment	0.002	2	0.001	0.206	0.814
	Treatment Day	0.004	1	0.004	0.700	0.404
	Hormone Treatment x Treatment Day	0.008	2	0.004	0.710	0.493
	Error	0.828	144	0.006		
Female 30 Day mass (g)	Hormone Treatment	0.008	2	0.004	0.515	0.599
	Treatment Day	0.007	1	0.007	0.960	0.330
	Hormone Treatment x Treatment Day	0.037	2	0.019	2.399	0.097
	Error	0.686	88	0.008		
Female 60 Day mass (g)	Hormone Treatment	0.004	2	0.002	0.100	0.905
	Treatment Day	0.018	1	0.018	0.974	0.328
	Hormone Treatment x Treatment Day	0.068	2	0.034	1.812	0.173
	Error	1.025	55	0.019		
Female 1 Year mass (g)	Hormone Treatment	0.062	2	0.031	0.174	0.913
	Treatment Day	0.128	1	0.128	0.719	0.853
	Hormone Treatment x Treatment Day	0.023	2	0.174	0.066	0.622
	Error	4.803	27	0.066		
Male Hatchling mass (g)	Hormone Treatment	0.001	2	0.001	4.865	0.030*
	Treatment Day	0.003	1	0.003	1.156	0.320
	Hormone Treatment x Treatment Day	0.002	2	0.001	1.386	0.256
	Error	0.053	83	0.001		
Male 30 Day mass (g)	Hormone Treatment	0.081	2	0.041	7.447	0.002*
	Treatment Day	0.010	1	0.010	2.461	0.099
	Hormone Treatment x Treatment Day	0.027	2	0.013	2.461	0.194
	Error	0.202	37	0.005		
Male 60 Day mass (g)	Hormone Treatment	0.054	2	0.027	0.828	0.454
	Treatment Day	0.036	1	0.040	1.178	0.293
	Hormone Treatment x Treatment Day	0.054	2	0.027	0.829	0.453
	Error	0.559	17	0.033		
Male 1 Year mass (g)	Hormone Treatment	92.907	2	46.453	1.961	0.166
	Treatment Day	5.250	1	5.250	1.491	0.236
	Hormone Treatment x Treatment Day	20.671	2	10.336	1.982	0.163
	Error	333.535	21	15.883		

Table 2: Two-way ANOVA results measuring mass for *Anolis sagrei* lizards treated with one of three Hormone Treatments: Estradiol, Testosterone, or Control (Ethanol). Hormone Treatments were applied at one of two different Treatment Days: Day 0 Post-Oviposition or Day 15 Post-Oviposition. An asterisk indicates a significant difference ($p < 0.05$).

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CHAPTER IV

EFFECTS OF EMBRYONIC EXPOSURE TO STEROID HORMONES IN SUBSEQUENT REPRODUCTIVE AND AGONISTIC ENCOUNTERS FOR *ANOLIS SAGREI* AS ADULTS

4.1 Abstract

Courtship and aggression are two social behaviors that are governed by specific sex steroid hormones either in an organizational capacity, or as activators. In this study we investigated the behavioral effects of Estradiol (E_2) and Testosterone (T) when applied at two different time points during embryonic development. We found that females treated with E_2 immediately after oviposition and midway through incubation were statistically more likely to present aggressive behaviors than controls. In addition, we found that E_2 treated females tended to use dewlap extensions more frequently in agonistic encounters compared to controls than T treated females. Treated females were no different than controls when examining courtship encounters. Treated males were not found to be behaviorally any different than controls in courtship and agonistic encounters. These results are indicative that E_2 plays an important role in female agonistic behavior. More importantly, it is involved in the organization of these behaviors during development, and potentially activated at a later point by naturally circulating hormones.

4.2 Introduction

There are a variety of cues that can be used between two individuals. These cues can come in a variety of modalities such as visual, vocal, and olfactory, for example. No matter the modality, there is an accompanying, frequently stereotyped, behavior. When observing courtship or aggression, it often is of interest whether that behavior is a learned or innate trait, or some combination of both. Learned behaviors, such as the species-specific song of birds, are disrupted in the absence of parental care because juveniles mimic adults (Mooney 2020, Rouse Jr. 2022).

In the case of the lizard species, *Anolis carolinensis*, the reproductive/aggressive display action pattern, a visual social cue, is an innate behavior. It is developed through the physiological mechanisms that encompass brain organization and later hormonal activation. These behaviors are not learned behaviors, as anoles do not give parental care to their offspring and hatchlings have been observed to perform displays within hours of hatching (personal observation). *A. carolinensis* lizards are precocial (Lovern and Jenssen 2003). The display action pattern of *A. carolinensis*, which is one of the most well studied of the anole species, is highly consistent across individuals. Anoles have three main categories of displays: push-up displays, dewlap extensions, and head-bob displays. The head-bob displays in *A. carolinensis* can be further subdivided into A, B, and C displays. Males tend to use C displays in courtship contexts or non-directed displays/long range displays (Jenssen et al. 2000). When males perform directed displays towards other males, they use predominantly A and B displays that increase in frequency as they get closer to one another (Jenssen et al. 2000). Females use these displays in different

contexts. C displays are used in agonistic encounters whereas A and B displays are used in courtship contexts (Lovern and Jenssen 2003). The X displays references the mixture of A and B displays seen in juveniles before sexual maturity; such displays indicate that although the communication signals do not require visual learning, they do undergo physiological refinement through development (Lovern and Jenssen 2003).

Anolis sagrei lizards on the other hand, show extreme variability in their display action patterns (Partan et al. 2011). Even though there is variability in displays, there is a higher social response of *A. sagrei* to the "signature pattern" defined by Scott (1984). Three signal types included in this signature pattern can be found; head-bobs (an up and down movement of the head), push-ups (an up and down movement of the body and sometimes body and tail—it may be a two-leg push-up of the anterior portion of the body or an entire-body push-up including the tail), and dewlap extensions (extension of the colored flap of skin found on the ventral portion of the neck) (Driessens et al. 2013). In addition to these three main signals, in agonistic encounters a dorsal crest and sagittal expansion behavior can be observed (Wade 2011). A dorsal crest is the raising of the dorsal surface along the back and neck of the animal into a peak or crest. As with many anoline species, a display difference between the sexes and between breeding and non-breeding seasons exists. *A. sagrei* males perform more dewlap extensions than females and juveniles (Partan et al. 2011). Dewlap extension frequency is variable within seasons, and is much more frequent during the breeding season, suggesting a prominent role in the species' courtship behavior. The use of head-bobs however, remains constant across all groups in the breeding season (Partan et al. 2011).

With each of these behaviors is an accompanying hormone regulator. Testosterone (T) can affect the propensity for a male to perform a dewlap extension display (Tokarz et. al 2000). For example, it was shown that castrated males treated with T were statistically more likely to exhibit dewlap extension displays than castrated controls (Rosen and Wade 2000, Winkler and Wade 1998). Further evidence has been provided through observations that more display behaviors are seen during the breeding season when T is high than the non-breeding season when T is low (Husak et al. 2007). In green anoles, T has been shown to be one of the most important activators of courtship and aggression behaviors, with Estradiol (E_2) having little effect aside from increased mounting behaviors (Wade 2011).

In this study, we investigated reproductive and aggressive display behaviors of *Anolis sagrei*, or the Cuban Brown Anole. With many studies having been done on the green anole, a species that is not a "close" relative with respect to the vast *Anolis* genus, results we obtain can be used to see if comparisons can be made among anoles as a whole. We examined the effects that T and E_2 have on reproductive and aggressive display behaviors. Our approach was to examine whether embryonic exposure of T and E_2 would affect the prevalence of the main display behaviors in adult males and females. To dive deeper into the hormonal mechanisms that drive the organization of such behaviors, we chose to test two time points of embryonic exposure. The first is indicative of maternal hormone influence while the second is indicative of embryonic gonadal hormone influence on the development of these individuals. We predicted that T exposed individuals from both time points would display increased "male" behaviors such as dewlap extensions, and E_2 exposed individuals from both time points would be no

different from the controls outside of mounting behaviors if hormonally mediated behaviors of the Brown Anole, *A. sagrei*, can be compared to hormonally mediated behaviors of the Green Anole, *A. carolinensis*.

4.3 Methods

Procedures described below were approved by the OSU IACUC (AS-17-8)

4.3.1 Females

Forty-eight *Anolis sagrei* females were acquired from field collected populations through a commercial supplier (Underground Reptiles, Deerfield Beach, FL). These females are the same as from Chapter III. Subjects were housed in 29-gallon group housing aquaria (3-5 females per tank). Tanks were filled with moist peat-moss, perches (wooden dowels), rock hides, and nest boxes filled with damp peat-moss for egg laying.

4.3.2 Egg Collection

In reproductively active female anoles, a single-egg clutch is oviposited every 7-14 days from an alternate ovary with each successive oviposition (Lovern and Wade 2003). Tanks were checked every day for the presence of a new egg. Due to group housing, eggs could be tracked to a specific tank, but not an individual mother. When eggs were found, they were randomly assigned to a treatment group and processed prior to incubation. Because females can store sperm from previous matings, we assumed

mating had already occurred in the field, so males were not necessary for co-housing with our females in the laboratory.

4.3.3 Egg Processing and Treatment Application

A. sagrei eggs (N=182) were collected from group-housed females and randomly assigned to different treatment groups. Upon collection, eggs were measured for mass before treatment. To treat eggs, a solution of .5µg/µL of T or E_2 (total dose ~2.5µg) using a 95% ethanol vehicle was prepared based on previous similar experiments (Lance and Bogart 1994, Warner et al 2017). This dosage was chosen based upon dosage amounts of similar experiments adjusted for the average mass of *A. sagrei* eggs. This method of hormone application has been shown to be effective (Crews, 1996; Warner et al. 2017). Eggs were treated at one of two time points (day 0 or day 15), based on treatment assignment. Dosing was accomplished by adding the hormone solution directly onto the outside of the egg using a 25-µL Hamilton syringe. The two separate time points chosen test the effects of hormones on developing embryos before and after gonadal development. Controls were given a solution of 95% ethanol containing no hormone. Once eggs were collected, they were placed into a jar containing a 1:1 mass:volume mixture of horticultural vermiculite and deionized water that was then sealed with plastic wrap and a rubber band in order to retain moisture. Eggs were placed at random into an incubator set at ~28° C to develop for ~30 days.

4.3.4 Hatchling Processing

The jars with the eggs in the incubator were checked daily for new hatchlings and incubation duration was recorded. One-hundred twenty viable hatchlings were obtained. Once a hatchling was found, it was measured for mass and SVL. Hatchlings were then toe clipped for a unique ID (Figure 7). Post-processing, male hatchlings were placed individually into 10-gallon aquaria and females were placed into 29-gallon group housing aquaria (3-5) females per tank) and raised until 1 year of age. Females were grouped within the same treatment hormones: Control (ethanol), Estradiol (E_2), and Testosterone (T).

4.3.5 Behavioral trials

Sixty-three *A. sagrei* hatchlings survived to adult age. Adults were tested at ~13 months of age, when they had reached reproductive maturity. Adults were sexed by checking for the presence or absence of post anal scales (Figure 6). One month prior to testing, females were moved to single housing. Each anole was subjected to different conspecific interactions including both intrasexual and intersexual interactions. For testing at 13 months, each anole's 10-gallon aquarium was moved to a neutral location where behavior could be monitored with minimal observer interference. Each treatment individual was paired for with two novel unrelated control individuals brought into the laboratory for the behavioral trials, one same sex conspecific and one opposite sex conspecific. Each anole was allowed to interact in the same aquarium for 10 minutes per trial (unless ended early due to fighting or mating) with the two novel conspecifics separately. Data from direct observations of trials were recorded by hand. Behaviors observed and quantified were head-bobs, pushups, dewlap extensions, dorsal crests, sagittal expansions, approaches, retreats, attacks, and mating. Aggression and

reproduction scores were calculated as the sum of the frequency of each behavior weighted by its point value. The point values assigned for aggression scores were based on Lovern and Jenssen (2001), with greater point values for behaviors that are more likely to occur only in the presence of a conspecific (Table 3). Point values were for reproduction scores (Table 4).

4.3.6 Data Analyses

The sample size of all groups was unequal due to deaths during upbringing which may have effect on our data analyses. Males and females were analyzed separately to account for differences in behavior due to *A. sagrei*'s sexually dimorphic nature. Total aggression scores and reproduction scores were calculated following modified chart values of prior studies (Lovern and Jenssen 2001). A two-way ANOVA (Hormone Treatment x Treatment Day) was used to test the interaction of our treatment groups as well as main effects of each on aggression and reproductions scores as well as individual behaviors of each type of encounter. Tukey *post hoc* tests followed in instances when $P \leq 0.05$. Statistical analyses were all performed using IBM SPSS software.

4.4 Results

4.4.1 Aggression Scores

Hormone Treatment had a significant effect on aggression scores for females (Two-way ANOVA: $F_{2,32} = 14.472$, $p < 0.001$), but not Treatment Day ($F_{1,32} = 1.557$, $p > 0.05$) (Figure 22). The interaction of Hormone Treatment and Treatment Day for female

aggression scores was also not significant ($F_{2,32} = 1.060$, $p > 0.05$). Post-hoc comparisons revealed that females treated with E_2 were significantly more likely to be aggressive compared to controls ($p < 0.001$) than females treated with T compared to controls ($p > 0.05$). Within these aggressive behaviors, it was found that Hormone Treatment had a significant effect on the amount of Dewlap-Extensions expressed by females (Two-way ANOVA: $F_{2,32} = 4.470$, $p = 0.019$), but not Treatment Day ($F_{1,32} = 2.068$, $p > 0.05$). There was also a significant effect of the interaction of Treatment Day and Hormone Treatment on the number of Dewlap-Extensions observed in female aggressive interactions ($F_{2,32} = 3.743$, $p = 0.035$) (Figure 23). Post-hoc comparisons showed that females treated with E_2 on Day 0 Post-Oviposition were significantly more likely to perform Dewlap-Extensions when compared to controls ($p = .048$) than females treated with T compared to controls ($p > 0.05$). Male aggression scores showed no significant difference of Hormone Treatment or Treatment Day (Two-way ANOVA: Hormone Treatment $F_{2,17} = 0.247$, $p > 0.05$, Treatment Day $F_{1,17} = 0.002$, $p > 0.05$). There was also no significant effect of the interaction of Hormone Treatment and Treatment Day on aggression scores of males ($F_{2,17} = 0.423$, $p > 0.05$) (Figure 24).

4.4.2 Reproduction Scores

Hormone Treatment and Treatment Day did not significantly affect the reproduction scores for females (Two-way ANOVA: Hormone Treatment, $F_{2,32} = 1.755$, $p > 0.05$; Treatment Day ($F_{1,32} = 0.732$, $p > 0.05$) (Figure 25). The interaction of Hormone Treatment and Treatment Day for female reproduction scores was also not significant ($F_{2,32} = 0.081$, $p > 0.05$). Male aggression scores showed no significant difference of Hormone Treatment or Treatment Day (Two-way ANOVA: Hormone

Treatment $F_{2,17} = 1.255$, $p > 0.05$, Treatment Day $F_{1,17} = 0.022$, $p > 0.05$). There also was no significant effect of the interaction of Hormone Treatment and Treatment Day on aggression scores of males ($F_{2,17} = 0.928$, $p > 0.05$) (Figure 26).

4.5 Discussion

4.5.1 Sex Steroid Hormones and Behavioral Encounters

We predicted that *A. sagrei* adults, both male and female, exposed to T at both embryonic time points would exhibit an increase in male behaviors such as Dewlap-Extensions in agonistic and reproductive encounters (Partan et al. 2011). This prediction was based on evidence that males that have higher T tend to be more successful in their territorial and reproductive encounter, in which dewlap extensions are frequently used. Additionally, we hypothesized that E_2 treated individuals would tend to show an increase in attempted mounting behavior when compared to controls if *A. sagrei* behavior could be compared to that of *A. carolinensis*.

In many species, social encounters are facilitated or hindered by exposure to or inhibition of sex steroid hormones, and sex steroid hormones are influenced by a variety of factors such as size, seasonality, and presence or absence of conspecifics and neighbors. In green anole lizards, T is an important activator in courtship and copulation, and E_2 plays a role in mounting behavior (Latham and Wade 2010). T increases the number of dewlap extensions in *A. sagrei*, and E_2 in connection with dihydrotestosterone has been shown to be vital in male courtship behavior (Tokarz et al. 2002, Crews et al. 1978). Although the effects of these hormones on behaviors have been studied in our model species, *A. sagrei*, studies have mostly consisted of altering the hormones of adult

lizards. These studies have revealed important information on how hormones activate certain agonistic and copulatory behaviors, but as far as we know, there are no studies looking at the relationship of E_2 and T as behavioral organizers during development in *A. sagrei*.

4.5.2 Roles of Estradiol and Testosterone in Agonistic and Reproductive Encounters of Anolis Sagrei

The results of the present study indicate that female anole lizards with elevated E_2 exposure during both early and mid-embryonic development had higher aggression scores than control females. However, sex steroid exposure during both early and late embryonic development had no effect on female reproductive encounters or any male encounters whether agonistic or reproductive. Though our study investigated E_2 as a behavioral organizer, a study on female Spiny Lizards, *Sceloporus jarrovi*, revealed that E_2 given to castrated females led to increased aggression in those females (Woodley et al. 2000). This indicates that E_2 is an activator of aggressive behaviors, and as such, is likely an organizer of them as well. With females that received embryonic treatments of E_2 having higher aggression scores, we wanted to investigate the individual behaviors to see if these more aggressive females tended to use one behavior more than others. These results are contrary to what we predicted with our hypotheses, as we assumed elevated T would lead to more aggressive behaviors between groups and E_2 would lead to more mating as had been seen in similar experiments with Green Anoles (Winkler and Wade 1998; Rhen and Crews 2000). This is due to evidence that T can permanently masculinize

the muscles and areas of the nervous system responsible for adult sexual displays (Wade 2012). Our results indicate that the organization pathways of Brown Anoles are potentially different than Green Anoles. Since these two anole species are not very closely related evolutionarily, this is not surprising.

Interestingly, when comparing the different treatment groups, those with higher aggression scores when compared to controls were not more likely to have a higher behavioral account in behaviors other than dewlap extensions. What we discovered was that females treated with E_2 on Day 0 post-oviposition were significantly more likely to use dewlap-extensions compared to controls and there was no effect of embryonic exposure of T on any behaviors of adults. These results are again, contrary to what was expected, as a study done on *A. sagrei* showed that T implants in castrated adult males resulted in a significant increase in dewlap-extension frequency (Tokarz et al. 2002). These contradicting results indicate that T serves more as an activator of anole behavior, at least in terms of dewlap extensions, than it is an organizer which has been observed to be true in *A. carolinensis* (Lovern et al. 2000). It is also possible that E_2 is an organizer of this behavior in females alone, as *A. sagrei* is a sexually dimorphic species, and different mechanisms are responsible for the behavioral organization of males. Evidence in support of this in a study on the Leopard Gecko, *Eublepharis mascularis*, where E_2 had an activational effect on sexual receptivity in females and not males suggesting sex differences in organization (Rhen and Crews 2000).

4.5.3 Future Implications

Our findings show that E_2 may be an important behavioral organizer in female agonistic behavior. In our study however, we tried to use a low dose of hormone when treating our eggs. This runs the risk of not being enough to permeate through the egg shell in a way that can reach the yolk and therefore influence development. There is also the possibility that the behaviors we investigated require hormonal activation. Future studies may try using a more invasive application method for delivering hormones, such as piercing the yolk for direct deposit of hormones in the vehicle of choice. They may also want to apply these hormones at the adult age to test if the behaviors need both hormonal organization as well as activation. If it is found that higher doses do have an impact, we can then begin investigations into how the toxicology of certain environments may end up affecting the reproductive output of *A. sagrei.* Due to the prevalence of endocrine disrupting chemicals in aquatic environments, some studies have noted that the resulting offspring of exposed individuals may be negatively affected (Bhandari et al. 2014). With *A. sagrei* being a species that reproduces near these aquatic environments and a generalist organism that has infiltrated urban environments including areas near water treatment plants, which falls under those aquatic areas that contain endocrine disrupting chemicals, more studies will need to be conducted to monitor the implications (Tiatragul et al. 2017, Bhandari et al. 2014).

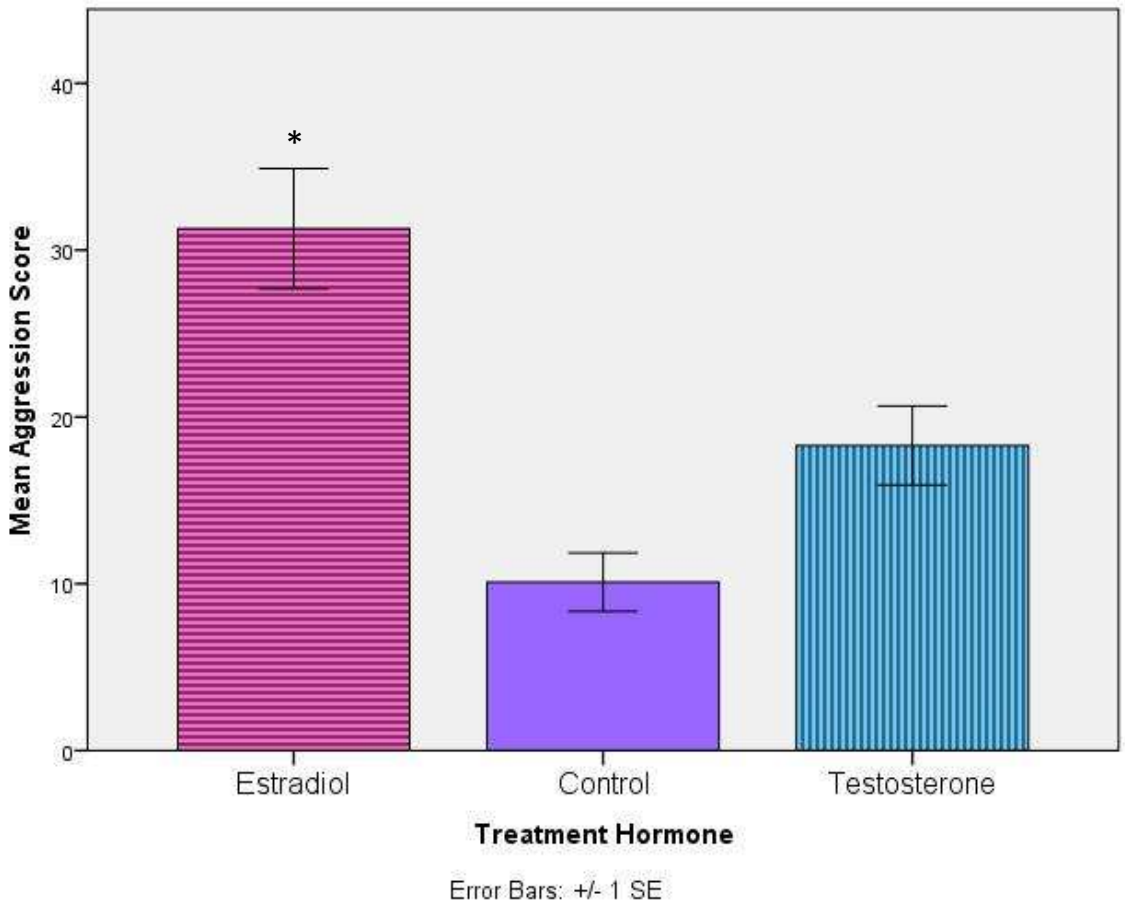

Figure 22: Mean (±SE) aggression scores of female *Anolis sagrei* by treatment hormone: Estradiol, Testosterone, and Control (Ethanol). Aggression scores are calculated by summing the number of behaviors (each assigned individual point values) seen during an agonistic encounter. The behaviors observed for calculating aggression scores are: Head-bobs + push-ups, dewlap-extensions, dorsal-crests, sagittal-expansions, approaches, retreats, and attacks. An asterisk indicates a significant difference ($p < 0.05$).

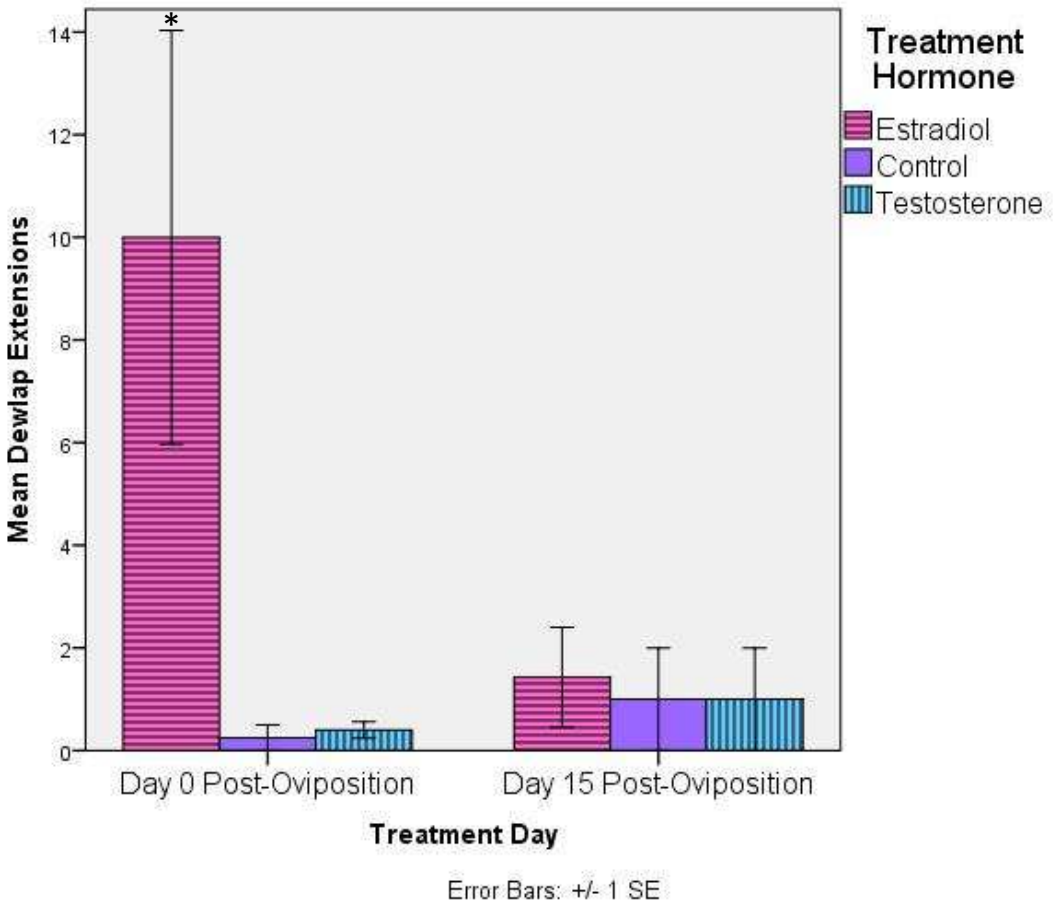

Figure 23: Mean (±SE) number of dewlap-extensions observed by females during agonistic encounters by Treatment Hormone and Treatment Day. An asterisk indicates a significant difference ($p < 0.05$).

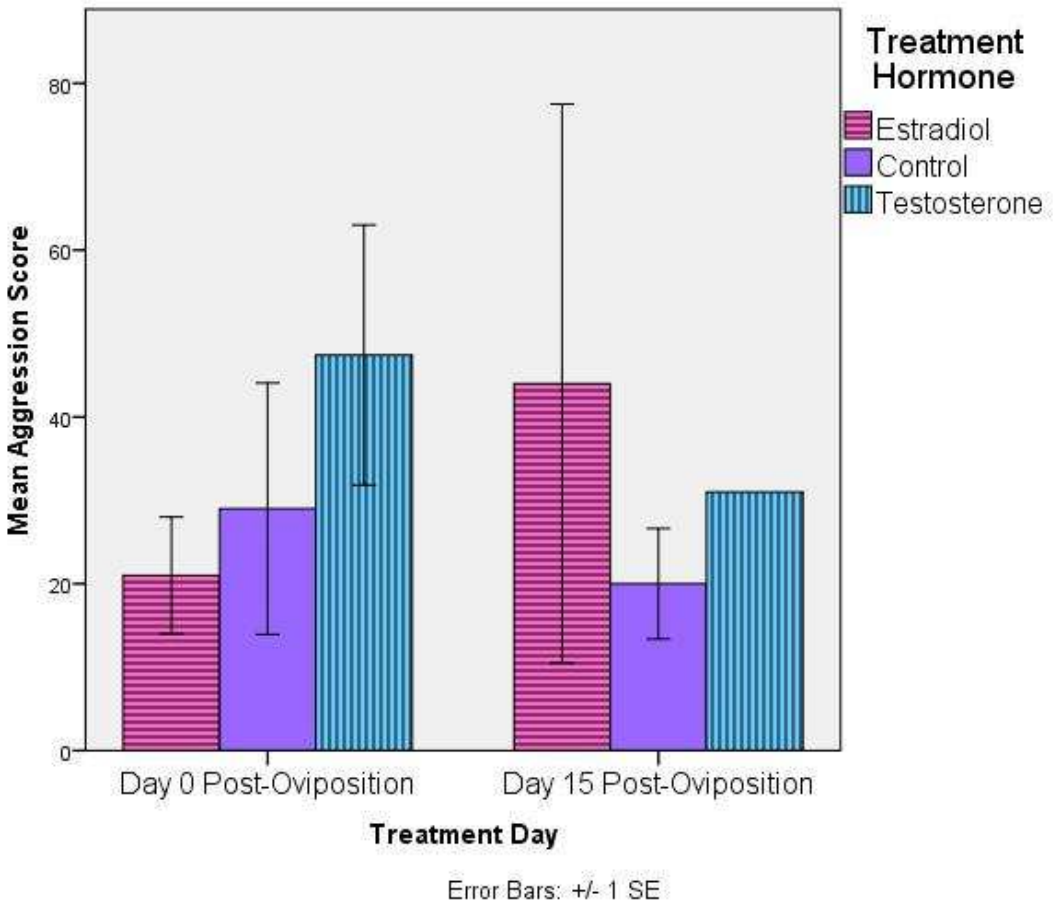

Figure 24: Mean (±SE) aggression scores of male *Anolis sagrei* by treatment hormone: Estradiol, Testosterone, and Control (Ethanol). Aggression scores are calculated by summing the number of behaviors (each assigned individual point values) seen during an agonistic encounter. The behaviors observed for calculating aggression scores are: Head-bobs + push-ups, dewlap-extensions, dorsal-crests, sagittal-expansions, approaches, retreats, and attacks.

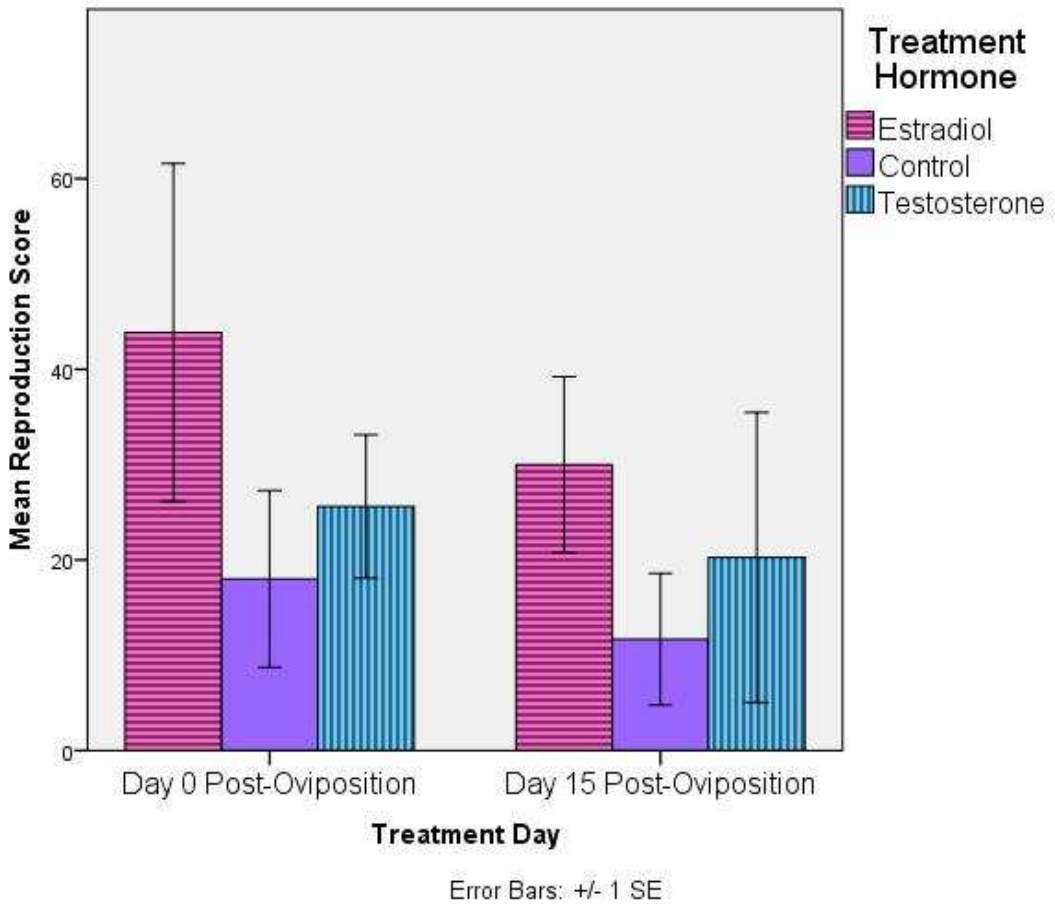

Figure 25: Mean (±SE) reproduction scores of female *Anolis sagrei* by treatment hormone: Estradiol, Testosterone, and Control (Ethanol). Reproduction scores are calculated by summing the number of behaviors (each assigned individual point values) seen during a courtship encounter. The behaviors observed for calculating reproduction scores are: Head-bobs + push-ups, dewlap-extensions, approaches, retreats, and mating.

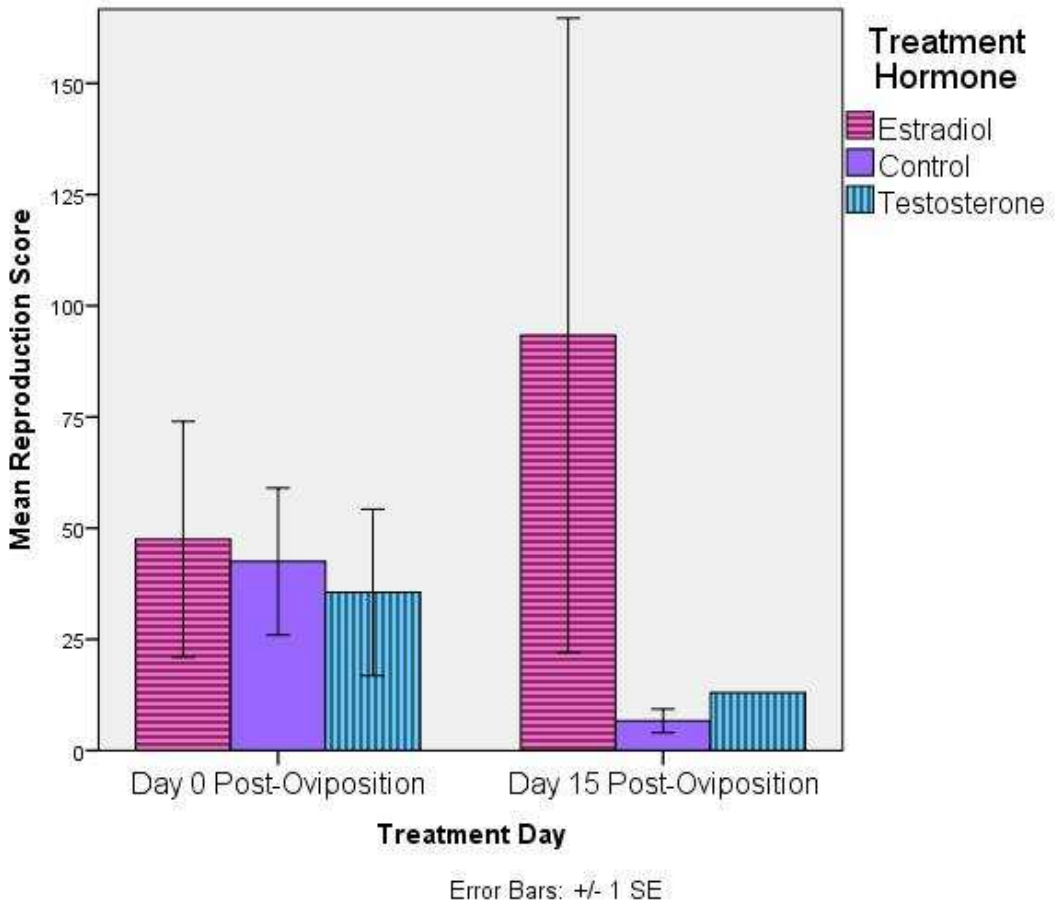

Figure 26: Mean (±SE) reproduction scores of male *Anolis sagrei* by treatment hormone: Estradiol, Testosterone, and Control (Ethanol). Reproduction scores are calculated by summing the number of behaviors (each assigned individual point values) seen during a courtship encounter. The behaviors observed for calculating reproduction scores are: Head-bobs + push-ups, dewlap-extensions, approaches, retreats, and mating.

Aggressive Behavior	Points Assigned Per Expression
Dewlap-Extension	1
Head-Bob + Push-Up	2
Dorsal Crest	3
Sagittal-Expansion	3
Approach	4
Retreat	-4
Attack	5

Table 3: Aggressive behavior chart for calculating aggression scores for *Anolis sagrei* agonistic encounters. Points are assigned for each behavior for each instance that behavior is observed within a set interaction time between to conspecifics.

Reproductive Behavior	Points Assigned Per Expression
Head-Bob + Push-Up	1
Dewlap-Extension	2
Approach	3
Retreat	-3
Mating	4

Table 4: Reproductive behavior chart for calculating reproduction scores for *Anolis sagrei* agonistic encounters. Points are assigned for each behavior for each instance that behavior is observed within a set interaction time between to conspecifics.

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VITA

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