THE INFLUENCE OF HORMONES AND NUTRITION ON COMMUNICATION SIGNAL DEVELOPMENT IN GREEN ANOLES (*Anolis carolinensis*)

By

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Abstract: Green anoles utilize highly stereotyped species specific displays for both intersexual and intrasexual communication. These displays have garnered much attention from researchers; however, to date no one has conclusively determined if or how anoles assess each other by these displays. Work on avian species, which also utilize stereotyped displays, has revealed that individuals that were exposed to a developmental stress produced lower quality displays. Additionally, testosterone (T) has been shown to either act as a developmental stress when levels are too high, or to mitigate a nutritional stress by either increasing foraging or differentially allocating nutrients. In the current study we assess whether a nutritional stress or treatment with either T or the anti-androgen flutamide (F) will increase or decrease stereotypy. We found that individuals exposed to a nutritional stress displayed with more stereotypy than those fed on a normal diet. Additionally, F treated individuals displayed with more stereotypy than controls while T treated individuals displayed with less stereotypy than either control or F treated individuals. We additionally found that our T treatment caused sub-adults to copulate, which has not previously been observed. Finally, juvenile T treated individuals headbobbed more frequently, but adult T treated individuals headbobbed less than their peers. We conclude from this study that while both the nutritional and hormonal treatment has a causal effect on headbobbing stereotypy, it seems unlikely that anoles use it as a mechanism to evaluate either opponents or mates.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

THE INFLUENCE OF HORMONES AND NUTRITION ON COMMUNICATION SIGNAL DEVELOPMENT IN GREEN ANOLES (*Anolis carolinensis*)

INTRODUCTION

In many mating systems males must fight with each other to gain access to females. These encounters can undergo a variety of forms, ranging from elaborate non-contact displays to physical fights in which one or both contestants can be injured or even killed (Ryan, 1980; Kelley, 2004). Green anoles are one species in which a large amount of assessment and, typically, fighting occurs (Crews & Greenberg, 1981; Decourcy & Jenssen, 1994; Jenssen et al., 2005; Tokarz et al., 2005). While green anoles have been studied extensively, with publications dating to the late 19th century (e.g., Monks, 1881), a complete understanding of exactly how they evaluate each other has remained elusive (Jenssen et al., 2012).

Organism Background

The green anole is a small lizard found throughout the southeastern United States (Lovern et al., 2004b). It has also proved to be adept at invading and establishing in new habitats as it has been introduced to several areas including the Hawaiian Islands and Taiwan (Latella et al., 2011). The green anole mating system is straightforward; males establish territories, and then mate with the females that inhabit their territory (Jenssen & Nunez, 1998; Jenssen et al., 2001). The defense and maintenance of a territory is directly related to the breeding success of an individual male, and only about one-third of males are capable of maintaining a territory.

Green anoles are well known for their rhythmic headbobbing displays, which are used to communicate intersexually and intrasexually (Figure 1). The breeding season runs from April to July, and it is during these months that most male-male agonistic interactions occur (Tokarz et al., 1998). The conflicts follow a highly ritualized pattern, during which either male may back down if it determines the risk of continuing the fight is not worth the rewards (Jenssen, et al. 1995a). Males begin by delivering a series of headbobs with increasing intensity. The headbobs are highly stereotypic and are classified as A, B or C display types depending on the number and timing of units (Decourcy & Jenssen, 1994; Lovern & Jenssen, 2003). During the early signaling phase, males will also laterally compress their body and erect a nuchal crest to appear larger. Additionally, a dark eyespot appears behind their eye, which has been demonstrated to be a potent agonistic signal (Larson & Summers, 2001; Korzan et al., 2006). If the interaction continues to escalate, the individuals will lock jaws with each other and attempt to throw the opponent to the ground. Jaw locking may continue for several minutes until one lizard backs down, or is too injured to continue. During the course of the interaction, there are several signals being sent and received, which may communicate either current physical state, or possibly some aspect of previous experience. Previous research has explored some of these signals, namely bite force, dewlap size and eyespot development (Lailvaux et al., 2004; Korzan et al., 2006; Husak et al., 2007; Cox et al. 2009). However, some of these signals have not been evaluated. The present study evaluates how nutritional and hormonal factors influence the stereotypy of the displays and the size of the dewlap.

Mating Displays

Highly stereotyped displays can be difficult to produce because of the neuromuscular requirements associated with them (Nowicki et al., 1998; Nowicki et al., 2002; Searcy et al., 2004; Searcy et al., 2010). If an individual lacked sufficient resources during critical periods of development, the body would be forced to allocate what resources are available to maintenance of current structures rather than building or strengthening new structures, particularly structures associated with signaling later in life (Nowicki et al., 1998; Brumm et al., 2009). If individuals can evaluate the stereotypy of opponent displays, it may be possible for them to gather information about physical condition, and by extension how much of a threat they pose.

Research on oscine song birds has demonstrated that juveniles placed under a nutritional stress produce songs that are lower in quality when compared to individuals fed a normal diet (Nowicki et al., 1998). Individuals that have higher amounts of parasites also produce inferior songs compared to uninfected individuals (Spencer et al., 2005; Bischoff et al., 2009). Finally, it has also been shown that females prefer males that produce more complicated songs, thus producing a causal link between juvenile nutrition and fitness later in life (Ballentine, 2004; Searcy et al., 2010). This relationship, known as the developmental stress hypothesis, has been well established in several species of oscine song birds, however it remains to be tested in other species which produce stereotyped displays rather than songs. By evaluating the developmental stress hypothesis in lizards, it may shed light on how, and if, males are evaluating each other during agonistic encounters. Additionally, since birds are phylogenetically nested within the reptile clade, it may shed light on the evolution of this particular method of competitor and mate evaluation.

Hormones could also influence the stereotypy of displays either directly, or by altering how the body uses resources that are available. For example, testosterone (T) influences the rate of displays in anoles (Tokarz, 1987; Lovern et al., 2001a). Additionally, it has been demonstrated that wild-caught juvenile green anoles that were administered T at approximately 60-90 days post-hatch displayed with more stereotypy than juveniles not treated with T (Pellerin & Lovern, 2005). To date however, Pellerin and Lovern (2005) is the only study which explored how T influences stereotypy in anoles, and they did not look at effects that may persist into adulthood. Fortunately, some studies have explored how various hormones influence song production in birds. T has been found to increase song size and repertoire (Van Hout et al., 2012). However, another study found that treating canary eggs with T may have induced a developmental stress as evidence by reduced growth (Muller et al., 2008). However, as adults, there were no differences in song quality when compared to controls. This apparent contradiction may be due to T acting as a developmental stressor, but simultaneously promoting song development that did not became evident until a later life stage (Muller et al., 2008).

Corticosterone (CORT) treatment has also been explored, and high levels of CORT will decrease song stereotypy later in life (Spencer et al., 2003). Previous work with anoles has demonstrated that when T is high, CORT is low, and vice versa (Tokarz et al., 1998). T may also decrease susceptibility of an individual to a nutritional stress either by increasing foraging aggressiveness or by altering how the body is able to use what resources are available to it (Pilz et al., 2004). T has been shown to alter activity levels in juvenile green anoles, which could result in increased foraging rates and increase the ability of juveniles to defend a home range (Stamps and Krishnan 1994b, Lovern et al., 2001b). Additionally, T has been demonstrated to alter how the body utilizes what resources are available to it, which could cause it to utilize resources in a more efficient manner (McCormick, 1999).

Dewlap Size

Anoles posses a dewlap, which is a sexually dimorphic structure that is located below the jaw, and dewlaps are frequently extended during displays. In green anoles, the dewlap is pink, and is much larger in males than it is in females (Jenssen et al., 2000). Currently, it is believed the dewlap is extended during some displays to increase conspicuousness to intended receivers of the signal (Simon, 2011). This hypothesis is strengthened by the observation that males extend the dewlap less frequently when closer to rival males (Jenssen et al., 2000). However, during courtship males commonly extend the dewlap when in close proximity to females, so it is possible that it serves another purpose in addition to increasing the conspicuousness of displays.

Recent work has demonstrated that individuals with higher T have larger dewlaps, and also bite with more force than individuals with lower T (Husak et al., 2007; Cox et al., 2009). Bite force has additionally been demonstrated to be an important factor in deciding which individual wins an encounter. It seems plausible that the dewlap may serve a dual function of both increasing the visibility of displays while also indicating the bite force of an individual. However, a study found that wild-caught individuals that were captured and placed under a nutritional stress bit with less force, but had the same-sized dewlap after 4 months of experiencing the nutritional stress (Lailvaux et al., 2012). While the study was well conducted, they did not start with hatchlings. It seems possible that dewlap size may have undergone the most growth before lizards were captured, or dewlap size may be decided by nutrient availability in a critical early window that was missed in that particular study.

Objectives

In the current study, both hormones (T exposure) and nutrition (food quantity) were manipulated in juvenile green anoles to determine what effects they had on both signal performance (stereotypy of displays) and associated morphological traits (mass, SVL and dewlap size). Specifically, juvenile green anoles were placed in either a normal or reduced feeding group until they reached 90 days of age. We then determined the stereotypy of their displays by calculating the coefficient of variation (CV) between displays. Additionally, these same lizards were exposed to either T, flutamide (T antagonist), EtOH (vehicle control) or left unmanipulated to determine what effect T exposure had upon stereotypy of displays, and if there was an interaction between nutritional state and hormonal state. We predicted that anoles given a normal diet would display with more stereotypy (lower CV) than juveniles on a reduced diet. Additionally, we predicted that normal fed anoles would have larger dewlap size compared to reduced fed individuals, when dewlap size is corrected for body size. Finally, we also predicted that T treated individuals would headbob with greater stereotypy than control individuals, and flutamide treated individuals would headbob with less stereotypy than either T or control individuals.

METHODS

Husbandry

All procedures were approved by the Oklahoma State University Institutional Animal Care and Use Committee (AS-10-8). Wild-caught, adult male and female green anoles were acquired from Candy's Quality Reptiles (La Place, LA) and were housed in the laboratory under conditions that have been documented to stimulate breeding and egg production (Lovern et al. 2004a). The wild-caught adults were housed in 110-L glass aquaria with a peat moss substrate, a nest box, water dish, a screen top, dowel rods and bricks for basking and hiding. Laboratory lights were regulated by a timer simulating the light/dark cycle found during the natural breeding season: 14 hours light to 10 hours dark for the overhead fluorescent lighting, 12 light to 12 dark for the UV lights (necessary for proper calcium metabolism), and 10 light to 14 dark for incandescent lamps (heat source). Laboratory temperatures averaged 25-28° C; however, in the cages temperatures ranged from 25-38° C due to the presence of the heat lamps at one end of the cage. Nest boxes were made from 0.5-L plastic containers filled with damp peat moss; these nest boxes provided a retreat in which females laid eggs. Cages were misted daily with water and lizards were fed four times per week on a diet of crickets (dusted with vitamin and mineral supplement), mealworms and waxworms. Each cage contained one adult male and a maximum of eight adult females.

Egg Treatment

Nest boxes were checked daily for eggs; when an egg was found it was weighed to the nearest mg and placed in a jar containing a 1:1 ratio of vermiculite to water (mass:volume) and covered with plastic wrap and a rubber band to ensure the substrate remained moist and the animal did not escape when it hatched. Prior to sealing the jar with plastic wrap, eggs were topically treated with 5 µg of T dissolved in 5 µl of 95% ethanol, 10 µg of flutamide in 5 µl of 95% ethanol (F), 5 µl of 95% ethanol (vehicle control), or they were left untreated. Treatments were placed directly onto the surface of the egg using a Hamilton syringe; previous research has demonstrated that T and other hormones are capable of crossing the shell and the hormone levels remain at an elevated level throughout incubation (Sargent and Lovern, Unpublished data). The treatment given was determined by a schedule to ensure an even and arbitrary distribution of treatments. After application of the plastic wrap, the eggs were placed in an incubator (Thermo Electron Corporation, Waltham, Mass.) set at a constant 28° C; at this temperature eggs typically hatch in about 34 days.

Hatchling Treatment

Upon hatching, juvenile lizards were weighed to the nearest mg, measured to the nearest mm and toe-clipped for individual identification. Hatchlings were then assigned to either a normal or reduced feeding treatment, based upon a schedule that ensured an even distribution of chemical treatments and sexes. Hatchings were placed in 110-L aquaria that were identical to those of the adults, except without a nest box and with the addition of artificial foliage. Each aquarium contained up to 12 individuals from the same nutritional treatment, but mixed chemical treatments. Juveniles were kept under the same lighting conditions as adults. Initially, the individuals in the normal feeding treatment received 15-30 mg of either crickets or mealworms daily per individual, while the reduced fed individuals received the same amount, but only three times a week. However, this feeding regimen proved to be too severe, as mortality was greater than expected. The feeding treatments were adjusted so that individuals in the normal feeding group received 30-40 mg of either crickets or mealworms per individual on a daily basis for the first 30 days, then 40-60 mg of food per individual from 30-60 days of age, then 60-75 mg of food per individual until 90 days of age. Individuals in the reduced feeding group received half that amount of food on a daily basis. When juveniles reached at least 45 days of age and at least 300 mg, they were given subcutaneous implants (as per original egg treatment). These implants function for an extended period of time; they are effective for at least two months (Lovern et al., 2004a). The implants were made by mixing either T or F in silicone and then extruding it through a syringe. The silicone was allowed to dry and was then cut in 2-mm sections. Blank implants contained only silicone. Each T implant contained approximately 1 mg of T; F implants contained approximately 2 mg of F. Implants were placed subcutaneously by making a small incision on the lower side of the abdomen slightly in front of the back leg. Animals were induced to effect with isoflurane by inhalation and then maintained on ice during the procedure. The incision was sealed with Vetbond. Animals were returned to their home cages within 60 minutes, after the effects of the isoflurane and ice completely wore off. Following 90 days of age, individuals were placed on an adult diet as described above.

Data Collection and Analysis

When juveniles reached 30 days of age, mass and snout-vent length were recorded again, and five individuals from varying treatments were placed in an observation aquarium.

Anoles were then video recorded for one hour using a high definition video camera (Sony HandyCam HDR-SR11) for later analysis. Through trial and error, we found that this method produced the most headbobs. The observation period for eliciting displays was repeated at 90 days of age.

Over the winter phase (from approximately November to March) in our colony, standard husbandry practices were followed (see above). In the subsequent breeding season (beginning in March), previous juvenile subjects that had now reached adult size were placed in an experimental aquarium and exposed to one or two stimulus animals of either sex to elicit headbob displays. Videos were analyzed, and each head bob was defined as A, B or C as outlined by Decourcy and Jenssen (1994). In addition, each display was analyzed frame-by-frame (1/30th-second interval). We calculated CVs for display units on an intra-individual basis. This yielded a CV value for each display unit for each lizard that gave more than one display. A lower CV score corresponds to a more highly stereotypical display. A similar method was used in Lovern and Jenssen (2003). This method was chosen because the number of headbobs a lizard produced would not skew the values. Avidemux software or windows media player was used for all video analysis.

We measured dewlap area by taking a photograph of the anole, with the dewlap manually extended, over a piece of graph paper for calibration purposes. Dewlap area was then calculated using ImageJ software. To make comparisons between individuals, the area of the dewlap was corrected by dividing by body mass.

All statistical analysis was performed using Minitab (version 14). To determine if there were any differences in hatching or survival among treatments, Chi-Square analysis was used. Because green anoles are sexually dimorphic for growth, males and females were analyzed separately. A repeated measures GLM was used to determine how individuals grew over the course of the study. One way ANOVAs were used to make all other comparisons among groups.

The EtOH and unmanipulated individuals were not different in any respect measured in this study, so they were also combined into a common control group. This combination left three normal fed treatments, Normal Control (NC), Normal Flutamide (NF) and Normal Testosterone (NT), and three reduced fed treatments, Reduced Control (RC), Reduced Flutamide (RF) and Reduced Testosterone (RT). For analysis of stereotypy, both males and females were analyzed together because males and females did not differ in stereotypy of displays and we were solely interested in the structure of the displays, not the motivation behind the displays. With respect to juvenile displays, only 90 of 402 displays were elicited from 30 day old individuals. Because there were not enough displays to extract any meaningful data, they were excluded from analysis. Because of this, all comparisons involving juvenile headbobs came from 90 day old individuals.

RESULTS

Ninety-seven eggs were collected in the summer of 2011, and 84 were collected in the summer of 2012, for a total of 182 eggs. Of these, 178 eggs successfully hatched (91%)

(Table 1). We found no significant differences in hatching success among the treatments (χ^2_4=0.399, P=0.983).

Post-hatching mortality was high throughout the experiment. Of the 165 hatchings, survival to 30 days was 65% (108 individuals), to 90 day was 42% (69 individuals), and to adulthood was 41% (67 individuals). For both the 2011 and 2012 breeding seasons, survival among chemical treatments did not differ statistically at 30 days (χ^2_4=0.400, P=0.982), 90 days (χ^2_4= 7.398, P=0.116) or adulthood (χ^2_4= 3.921, P= 0.417) (Table 2). Individuals fed a normal diet did not survive better than individuals fed a reduced diet at 30 days (χ^2_8= 4.049, P=0.853) 90 days (χ^2_8= 9.040, P=0.339) or adulthood (χ^2_8= 11.739, P=0.163) (Table 2).

Growth

Looking at all individuals, we found that individuals grew over the course of the study (Figure 2); age and individual ID were significant factors influencing mass for both males (repeated-measures GLM, age: $F_{2,61}$=178, P<0.001; ID: $F_{21,61}$=2.86, P=0.002) and females (age: $F_{2,51}$=113, P<0.001; ID: $F_{17,51}$=2.10, P=0.034). At hatching there were no significant differences in growth between any nutritional treatments for both the 2011 and 2012 breeding seasons ($F_{3,30}$=1.00, P=0.409). By 30 days, normal fed individuals were significantly heavier than reduced fed individuals from the 2011 breeding season ($F_{1,25}$=12.95, P=0.001), and this pattern persisted at 90 days ($F_{1,14}$=11.14, P=0.006). However, the same pattern was not observed for individuals from the 2012 breeding season at 30 days of age ($F_{1,10}$=.91, P=0.365) or 90 days of age ($F_{1,9}$=0.00, P=0.968). Further analysis revealed that the 2012 individuals (both normal and reduced fed) were equivalent to the 2011 normal fed individuals at 30 ($F_{2,12}$=.54, P=0.598) and 90 days of age ($F_{2,7}$=1.67,P=0.279). Because of this, we concluded that the 2012 nutritional treatment failed, and all individuals from the 2012 breeding season were treated as normal fed individuals.

Chemical treatment comparisons yielded no differences in growth at any age group, with the exception that NT males were significantly larger as adults compared to NF and NC males ($F_{2,5}$=18.77, P=0.020). However, this pattern was not observed for reduced fed individuals from corresponding treatments.

When we compared all male individuals from all chemical treatments and years together, we found that there were no differences between normal fed males and reduced fed males at hatching ($F_{1,76}$=1.35, P=0.250), but by 30 days the normal fed individuals were larger ($F_{1,52}$=18.33, P<0.000) and at 90 days this pattern continued ($F_{1,32}$=13.33, P=0.001). However, as adults there was no statistical difference between the two feeding groups ($F_{1,35}$=0.17, P=0.684). Females showed a similar pattern with no significant difference at hatching ($F_{1,84}$=1.97, P=0.165) but normal fed individuals were larger at 30 ($F_{1,51}$=10.22, P=0.002) and 90 days ($F_{1,35}$=14.94, P<0.000). When females reached adulthood, there was a significant difference, with the reduced fed individuals being larger ($F_{1,36}$=16.70, P<0.000).

Headbobbing Display Stereotypy

We recorded a total of 617 headbobbing adult displays from all treatments; of these, 352 were type C. Because we observed so few A and B display types, we limited our analyses to the C displays. There was no difference between males and females in regards to headbobbing stereotypy ($F_{1,5}$ =0.24, P=0.651), so they were combined for all statistical analysis.

Both feeding and chemical treatments influenced stereotypy of adult displays (Figure 3). Chemical treatment significantly affected stereotypy for the normal fed individuals ($F_{2,9}$=9.29, P=0.011), with NF treated individuals showing the greatest stereotypy between displays (mean CV=19.4%), NT treated individuals showing the least stereotypy (mean CV=40.2%) and NC treated individuals intermediate between the two (mean CV=29.2%). However, there was no difference in stereotypy between RC, RF and RT treated individuals ($F_{2,10}$=0.13, P=0.879). NC individuals headbobbed with less stereotypy than RC individuals, although this effect was only marginally significant ($F_{1,4}$=7.30, P=0.054), NT individuals displayed with much less stereotypy than RT individuals ($F_{1,6}$=46.67, P=0.001) and there was no difference between NF treated individuals and RF individuals ($F_{1,7}$=0.00, P=0.986).

Juvenile Stereotypy

The NC 90 day old juveniles headbobbed with the same stereotypy as the adult NC group ($F_{1,4}$=.00, P=0.962; Figure 4). Additionally, there was no difference between juvenile NT treated individuals and juvenile NC individuals ($F_{1,9}$=.32, P=0.586). However, NT treated juveniles headbobbed with significantly more stereotypy than NT treated adults ($F_{1,9}$=8.87, P=0.018). We were unable to collect enough F or reduced fed juvenile displays to perform statistical analysis.

Headbobbing rates

Adult males headbobbed at a greater rate than juveniles for all treatments pooled ($F_{1,66}$=9.09, P=0.004; Figure 5). Although T treated juveniles headbobbed at a higher rate than C or F treated juveniles, this effect was not significant ($F_{2,14}$=0.23, P=0.798). However, both NC treated juveniles ($F_{1,15}$=10.50, P=0.006), and the NF treated juveniles ($F_{1,8}$=11.21, P=0.012) headbobbed at a significantly lower rate than adults of the same treatment. However, there was no significant difference between NT treated juveniles and NT treated adults ($F_{1, 12}$=1.29, P=0.280).

Dewlap Size

When corrected for mass, adult males had a larger dewlap than adult females or juveniles ($F_{1, 31}$=110.77, P=0.000). Neither feeding treatment ($F_{1,14}$=1.05, P=0.325) nor chemical treatment ($F_{2,14}$=.80, P=0.471) significantly influenced dewlap size once individuals reached the adult stage. However, in 90 day old juveniles, treatment with T significantly increased dewlap size, while treatment with F significantly reduced dewlap size in both males ($F_{2,16}$=9.44, P=0.003) and females ($F_{2,8}$=18.31, P=0.003).

DISCUSSION

Hatching, Survival and Growth

The chemical treatments did not appear to affect the hatching or survival of individuals. Although T treated individuals did hatch and survive at a higher rate, this difference was not significant. We originally thought that T treated individuals would survive better, either because of more aggressive feeding behavior or possibly because T may cause differential allocation of resources. It is possible that T doesn’t help juvenile green anoles survive a nutritional stress in the same manner that has been reported in other studies.

However, it is possible the initial nutritional stress, which killed most of our early lizards, skewed the results. Finally, several lizards were able to escape, and since they died if they were not recaptured, we did not differentiate between lizards that were lost and died, so perhaps enough escaped to alter the results. Lastly, it is also possible the lizards were perishing from some other cause, unrelated to the study. This however, seems unlikely, because other experiments running at the same time in the same room didn't observe increased mortality. The nutritional treatment also did not affect survival; this is surprising since so many individuals died, presumably from lack of nutrition and because we did observe such a stark difference in growth between the two groups. As stated above, it is possible enough lizards escaped to prevent a significant difference from being found. There were, however, clear differences in the growth of individuals from 2011. All individuals hatched at approximately the same size. By 30 days, normal fed individuals were larger than reduced fed individuals, and this difference increased to 90 days as well. By spring, however, there were no differences in size between the two groups, so any differences in behavior are likely to result from previous body condition and not current body condition. A similar pattern was observed by Nowicki et al. 1998. The 2012 nutritional treatment was not as successful however, as we were unable to detect a statistical difference between normal and reduced fed individuals. If you will recall, our initial feeding treatment was too severe and many lizards were perishing. We slowly began to increase the feeding until survival was better. We then used the final feeding treatment for all of 2012. It appears in order to nutritionally stress green anoles, you have to feed at levels that will result in the death of some individuals.

Stereotypy

The nutritional stress had a clear impact on headbobbing stereotypy, just not the impact that was expected. We hypothesized that normal fed individuals would headbob with more stereotypy than reduced fed individuals. We hypothesized this because individuals developing in a less stressful environment should be able to allocate resources to development of the nervous and muscular structures needed to produced stereotyped displays, which may later increase fitness. Reduced individuals will have to allocate what resources are available to maintaining their physical state, rather than improving it. We found that NC treated individuals actually headbobbed with less stereotypy than RC individuals, and the same held true for NT and RT groups. NF and RF groups were not significantly different from each other, but that is likely due to the chemical and not nutritional treatment, and will be addressed below. There are two mutually exclusive conclusions: the first is that a less stereotypic display indicates a better nutritional history. This seems unlikely, as it would be easier to fake than a more stereotypical display. The second conclusion is that anoles do not evaluate prior body condition based upon stereotypy. It is possible that there is little need to understand an opponent's nutritional history in this group of animals if current body condition is the most likely predictor of a fight outcome. This is interesting, because previous research demonstrates that individuals raised under a nutritionally stressed environment bite with less force, and bite force has been demonstrated to be a good predictor of fight outcome (Lailvaux et al., 2004; Lailvaux et al., 2012). It is also possible that information about previous body condition is communicated through alternative means.

The chemical treatment responded exactly opposite to the predicted pattern as well. It was hypothesized that T treated individuals would show greater stereotypy than C or F treated individuals. In fact, we found that T treated animals produced the least stereotypic displays, while F treated individuals produced the most stereotypic display, with C treated individuals in between. This further points to the conclusion that stereotypy is not used by green anoles to evaluate a rival. While current T levels have been shown to influence the outcome of agonistic interactions in anoles and other species, to our knowledge no study has looked at how early T exposure influences fight outcome later in life (Rohwer & Rohwer, 1978; Husak et al., 2007). Further strengthening the conclusion that T decreases stereotypy later in life, the NF treated group, in which T receptors were blocked, showed the most stereotypic displays of all normal fed treatments. It is our conclusion that early T exposure decreases stereotypy later in life. Unpublished research indicates that adult exposure to T has no effect on stereotypy, so T must be acting within a critical window to decrease stereotypy (Haines et al., In Prep). In the current study, we found that treatment with T did not significantly affect stereotypy in juveniles, as T treated juveniles displayed with no more stereotypy than controls. However, it did not decrease their stereotypy either, as juvenile T treated individuals were more stereotypic than their adult counterparts. Unfortunately, we were unable to get enough displays to calculate stereotypy in F treated juveniles.

Headbobbing Rates

Previous research indicates that administering T causes an increase in aggressive behavior in both adults and juveniles (Tokarz, 1995; Lovern et al., 2001a). Headbobbing displays are one of the behaviors that increase when T is higher. This pattern held true for juveniles, but during the subsequent breeding season, the T treated individuals gave the fewest headbobs and had the lowest headbobbing rate. This contradiction is most likely explained by the anole's body attempting to return to homeostasis. When we artificially increased T levels in the juvenile anoles, it is speculated that the body attempted to reduce the effect of elevated T by down regulating androgen receptors and by decreasing endogenous T production. When the implants fell out or ran out of T over winter, what T the body produced was not sufficient to stimulate the animals, and they showed less courtship and aggressive behavior when compared to other treatments. This explanation could be easily tested by staining the brains of T treated anoles for androgen receptors, and merits exploration. Previous research has demonstrated that androgen receptors will increase in number in various regions of the body in response to varying levels of androgens found circulating in the blood (Kerr et al., 1995; Kumar and Thakur, 2004).

Dewlaps and other findings

We found that the chemical treatment had an influence on dewlap size. Individuals treated with T had larger dewlaps as juveniles; while F treated individuals had smaller dewlaps. C treated individuals were intermediary between the groups. This effect did not persist into adulthood. These results support previous work indicating that testosterone can increase dewlap size (and likely bite force), however the increase is not permanent (Irshick et al. 2006a; Husak et al., 2007; Cox et al. 2009; Lailaux et al., 2012).

Additionally of interest, we observed on two separate occasions, once in the fall of 2011, and once in the fall of 2012, sub-adult anoles engaging in copulation. In the authors' experience in both field and lab studies, this has never been observed previously, and we were unable to find any reference in the literature as well. The first time this occurred we were unable to collect any form of photographic evidence, and were only able to identify the male individual, who was treated with T. The second time, we were able to collect video evidence, and determine that the individual on top was a T treated male, and the individual on the bottom was a T treated female (Figure 6). Both occurred when the animals in question were between 90 and 97 days of age. Further examination revealed that the male individual had a swollen area just below his anal vent, which usually indicates sexual maturity (Figure 7). A swollen area below the anal vent was also observed on several other T treated juvenile males. It is likely that they exhibited this early sexual maturity because of the T treatment, and it is unlikely to occur in nature. However, there are several physiological events that could cause a sub-adult individual to have higher T levels than normal, and it could potentially confer a reproductive advantage to an individual that lives in an area where breeding is not temporally restricted, such as Hawaii where green anoles have been introduced.

Summary

We conclude that green anoles do not evaluate individuals based upon display stereotypy. Although it does appear that hormones and nutrition have a causal effect on stereotypy, the effect manifests itself in a manner that would easily allow cheaters. Namely individuals that came from the nutritionally stressed group actually displayed with more stereotypy than normal fed individuals. Additionally, T treated individuals displayed with the least stereotypy while F treated individuals displayed with the most stereotypy, with C treated individuals intermediate between the extremes. Based on previous research we hypothesized that a better nutritional history and T treatment would result in increased stereotypy. In terms of the developmental stress hypothesis, the results of this study do not suggest that the developmental history of green anoles influences their displays in a manner that would allow individuals to evaluate each other. This could be because interactions between anoles are not influenced by previous physical state, or another aspect of their display that was not evaluated in this study is used to convey this information. It is also possible that because anoles do not learn to display, like oscine song birds do, the developmental stress hypothesis doesn't apply to them. Additionally, it may be possible that current physical condition is more important than previous physical condition in terms of determining the potential of an individual to secure and defend a territory. We additionally found that if juvenile green anoles are exposed to high enough levels of T, they will begin to engage in reproductive behavior. While this could be a significant reproductive advantage, there may also be costs because our study also revealed that individuals exposed to high T early in life were less aggressive when exposed to a conspecific. Considering that green anoles differentially allocate T to eggs, it is interesting to consider the potential tradeoffs between the juvenile life stage and the adult life stage (Lovern and Wade, 2001).

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APPENDICES

Table 1 Total number of eggs collected and hatched for the 2011 and 2012 seasons.

Eggs Collected				**Eggs Hatched**			
Treatment	**2011**	**2012**	**Total**	**2011**	**2012**	**Total**	**Hatching Success**
EtOH	17	13	30	15	10	25	83%
F	33	28	61	30	23	53	87%
T	32	29	61	31	26	57	93%
Un	15	15	30	15	15	30	100%
Totals	97	85	182	91	74	165	91%

Table 2 Survival of hatchlings to 30 days, 90 days and into adulthood, with survival percent in parentheses.

Treatment	**30 Days**	**90 Days**	**Adulthood**
EtOH	17/25 (68%)	10/25 (40%)	9/25 (36%)
F	36/53 (68%)	19/53 (36%)	19/53 (36%)
T	36/57 (63%)	25/57 (44%)	25/57 (44%)
Un	19/29 (65%)	15/29 (52%)	14/29 (48%)
Total	108/165 (65%)	69/165 (42%)	67/165 (41%)

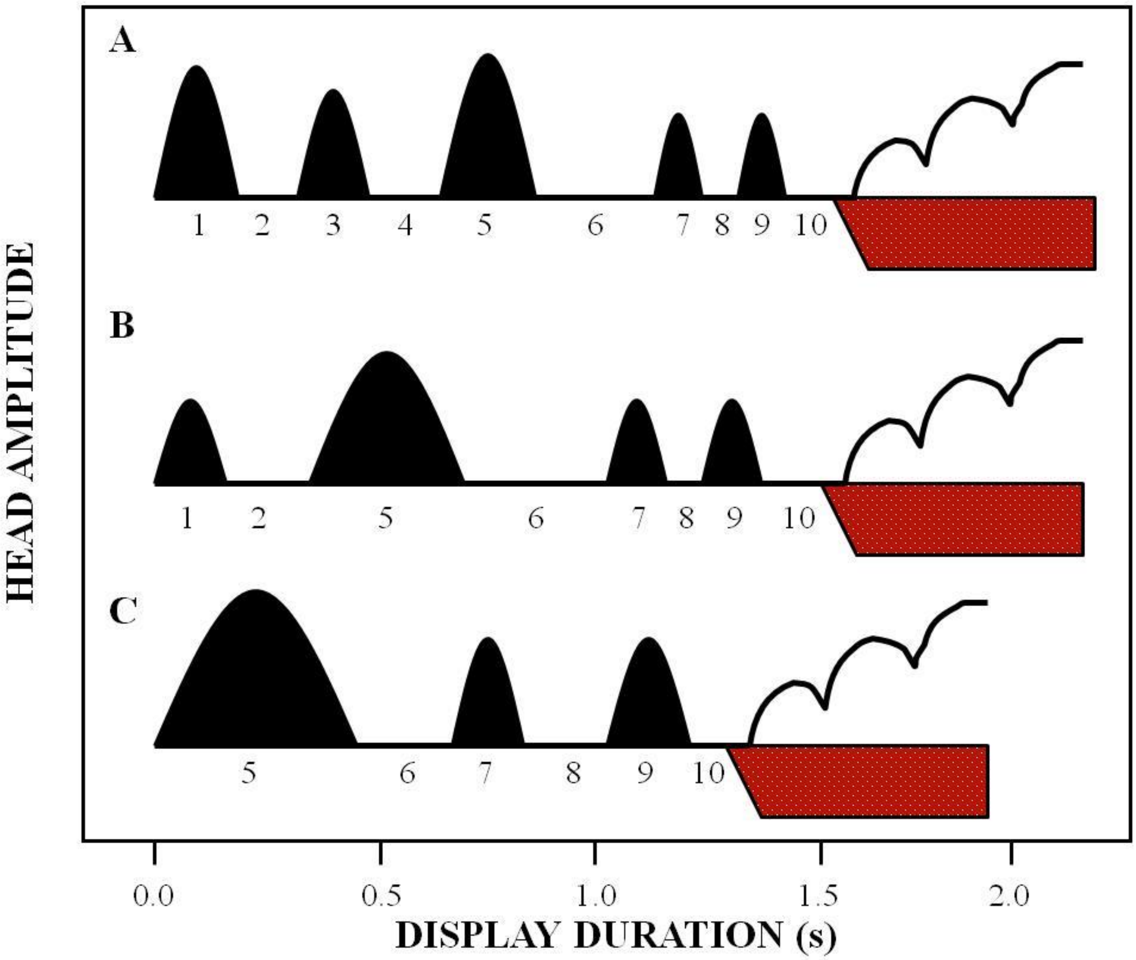

Figure 1: Display action pattern of the green anole headbob, bumps represent when the lizard's head is raised, spaces in between are when the lizard's head remains motionless. Red unit below line indicates optional dewlap extension. Stepwise bumps represent the shudderbob, a set of rapid, shallow bobs that can be from 1-10 units in length, from Lovern and Jenssen, 2003.

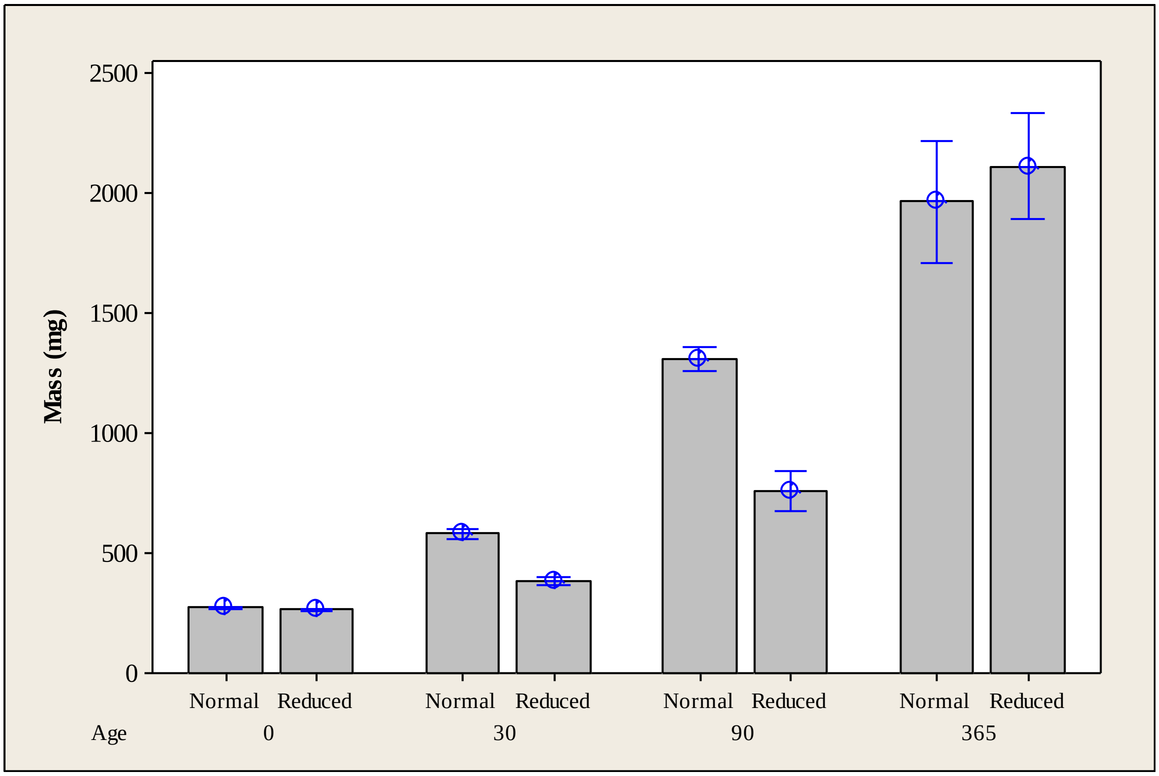

Figure 2: Average mass of normal and reduced fed individuals at hatching (0), 30 days of age, 90 days of age, and during the subsequent breeding season (365). There was no difference in mass at hatching, but by 30 days there was a significant difference between normal and reduced fed individuals, and this difference was strong at 90 days of age. After 90 days of age both treatments were fed the same amount of food and by the next spring their masses were not significantly different.

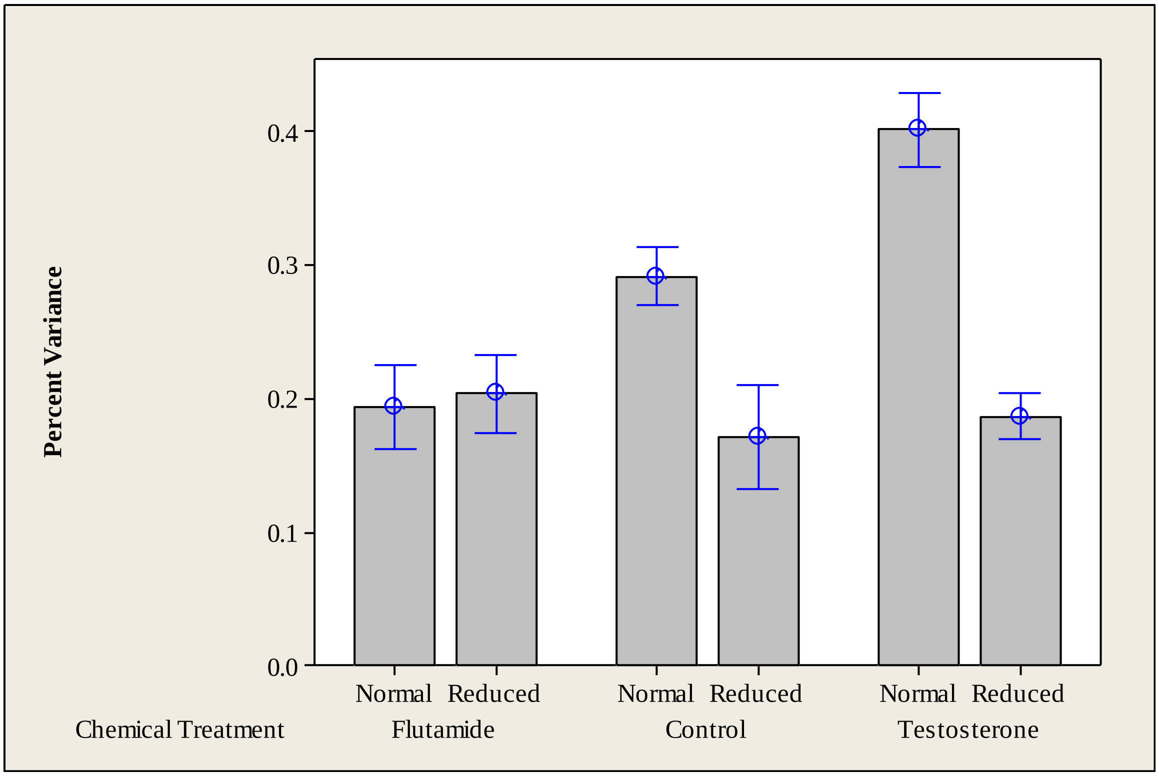

Figure 3: Variance in adult displays as measured by CV. A lower number represents a more stereotypic display. There were no significant differences between any of the reduced fed groups. There was a significant different between the normal groups, however, with F treated individuals exhibiting the most stereotypic displays and T treated individuals exhibiting the sloppiest displays; control treated individuals were intermediate.

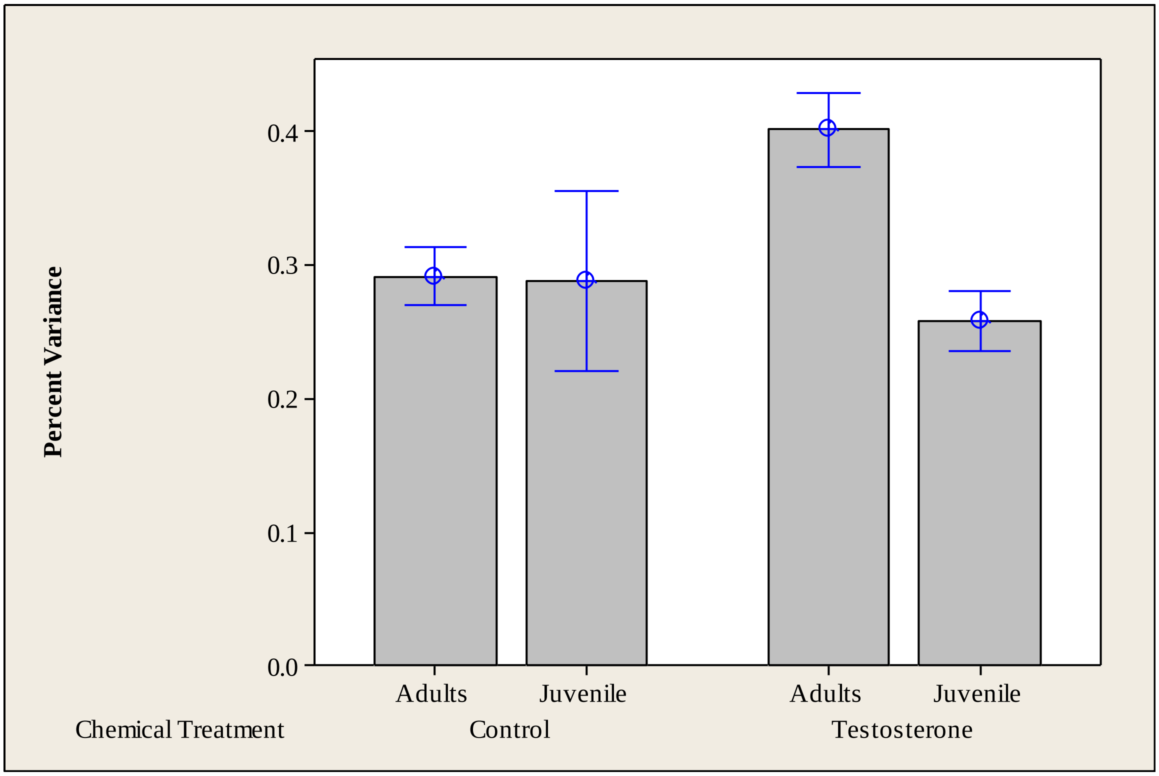

Figure 4: Stereotypy of 90 day old juveniles compared to adults. There were no differences between control juveniles, control adults or testosterone juveniles. However, there was a significant difference between testosterone adults and juveniles, as juveniles displayed with more stereotypy than adults.

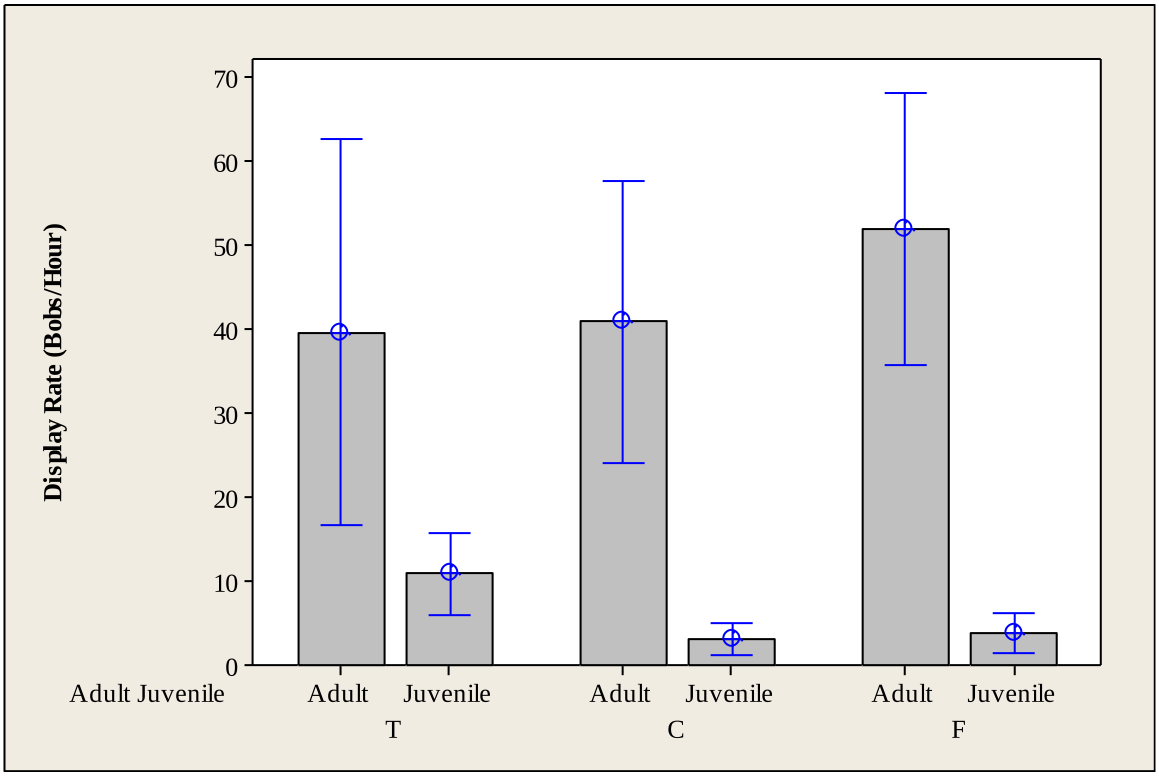

Figure 5: Headbobbing rates as measured in displays/hour. Adults headbobbed significantly more frequently than juveniles for the control and flutamide treatment, but not the testosterone treatment. Testosterone treated juveniles headbobbed more than any other chemical treatment for that age group (but not significantly so), while testosterone treated adults headbobbed the least out of any chemical treatment for that age group (but not significantly so). There were no significant differences between juveniles or adults with respect to treatment.

Figure 6: Two sub-adult juveniles copulating. The individual on top is a T treated male and the individual on the bottom is a T treated female. Both individuals are between 90-97 days of age.

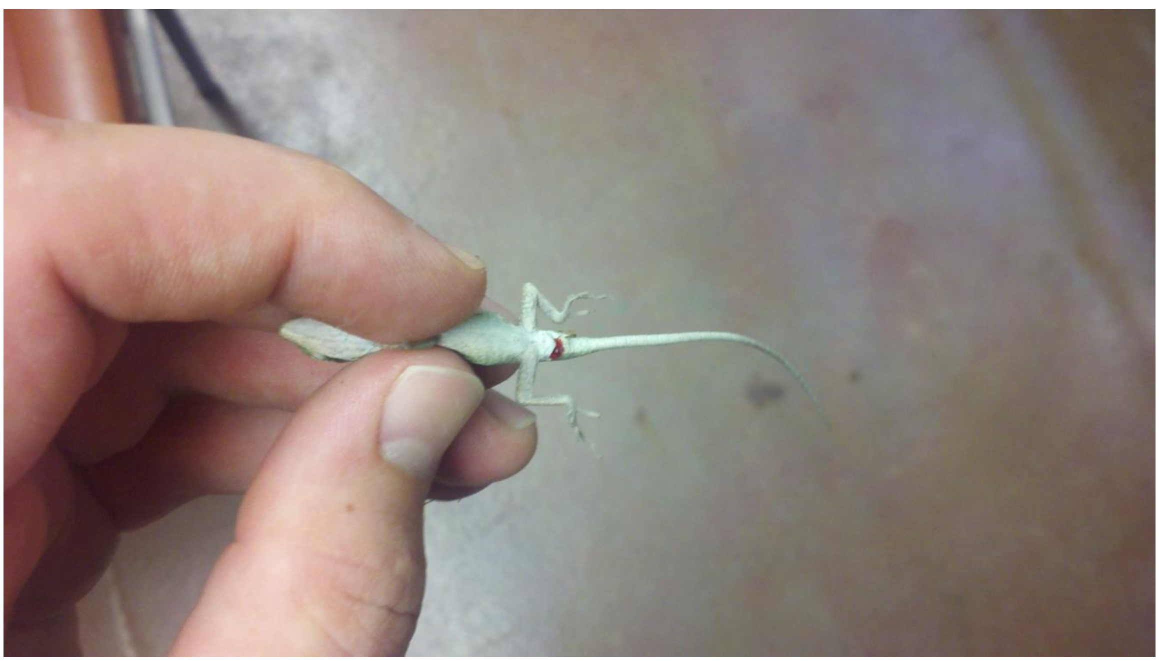

Figure 7: Sub-adult 91-day-old male that was testosterone treated. This individual recently copulated with another unknown individual from the study. You can see the hemipenis is still everted and the region of tail directly behind the anal vent is swollen.

VITA

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