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# The Evolution of Androgen Receptor Expression and Social Behavior in Anolis Lizards

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# THE EVOLUTION OF ANDROGEN RECEPTOR EXPRESSION AND SOCIAL BEHAVIOR IN *ANOLIS* LIZARDS

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## Abstract

Steroid hormones have a well-studied influence on behavior, but circulating levels of testosterone alone cannot fully predict levels of social, androgenic behaviors. Androgen receptor (AR) expression may bridge the gap between circulating androgens and the muscles that control social behavior: species with higher rates of behavior should have higher levels of AR protein in the nuclei of the muscles that control these behaviors. In anole lizards, the ceratohyoid (CH) muscle extends the dewlap, a colorful throat fan used in social displays, and the retractor penis magnus (RPM) muscle retracts the intromittent organ after copulation. I observed social behavior in the field and measured AR protein in the nuclei of the CH and RPM in male lizards of six anole species native to the island of Hispaniola: *Anolis chlorocyanus, A. coelestinus, A. brevirostris, A. distichus, A. cybotes,* and *A. longitibialis*. I used immunocytochemistry to measure muscle AR in each of ten individuals per species, and calculated species averages for AR expression. In the field, male anoles of these species showed substantial variation in both their average rate of dewlap display and their observed rate of copulation. I found that lizard species with higher rates of dewlap displays have marginally more AR in the muscle that controls these same dewlap extensions, but found no support for such a relationship in the copulatory system. Furthermore, there was no relationship between AR expression in one muscle and AR expression in the other. These results suggest that AR expression is independently controlled in muscles that control different social behaviors.

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I would like to thank my advisor, Dr. Michele Johnson, whose hard work and patience made this thesis possible. The last two years in her lab have been full of joy, and my experiences as a research student made me who I am today. From the moment she showed my Bio I class a picture of her research students working in the field, I knew I wanted to be a part of it. To have been able to is a privilege beyond words, and I owe her a great measure of my success at Trinity. Dr. Johnson once told me she had asked me to join her lab because I laughed at her husband's jokes. While I cannot claim to live up to Dr. Kevin McIntyre's excellent sense of humor, I have him to thank for introducing me to the lab. I would also like to thank Dr. Nancy Mills, whose invitation to join her research lab my freshman year propelled me into a world I had no previous connection with.

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## Introduction

Hormones are signaling molecules that are synthesized and secreted by endocrine organs such as the thyroid and adrenal glands, as well as by the vertebrate brain, that then travel through the bloodstream to act on tissues. By exposing the entire body to a hormone, organisms can respond to internal and environmental stimuli, facilitating a synchronized response in multiple systems at once. When a response is required in an organism, whether it is to restore homeostasis or to facilitate a mating display, a slight change in circulating hormone levels is often sufficient to initiate a tissue-level response in an individual. Through the action of hormones, organisms can control traits ranging from circadian rhythms and internal homeostasis, to higher-level systems such as stress and social behavior (Adkins-Regan 2005).

Hormones can be divided into several categories based on their biosynthesis: amino-acid derivatives, fatty-acid derivatives, peptide hormones, and steroid hormones. Steroid hormones generally possess the same basic chemical structure— even across the animal kingdom (Adkins-Regan 2005). All steroid hormones are derived from cholesterol, and thus possess the same basic carbon backbone: three hexagons and a pentagon (Carson-Jurica et al. 2008). Differences in functional groups and oxidation states then define each unique steroid hormone, resulting in differences to their active sites and binding affinities (Adkins-Regan 2005, Carson-Jurica et al. 2008). In general, five primary classes of steroid hormone exist: mineralocorticoids, glucocorticoids, progestogens, estrogens, and androgens (Miller 1988, Tsai & O'Malley 1994). Androgens, including testosterone, are traditionally considered the "male hormones," and work through binding to androgen receptors (AR) in cells, resulting in a cascade of changes in gene expression (Adkins-Regan 2005).

Androgen receptors are nuclear transcription factors. When AR is first synthesized, it must be phosphorylated to lend the protein greater affinity for its ligands, namely testosterone and other androgens (Brinkmann et al. 1999). When an androgen then binds to AR, this provokes a conformational change that induces an additional phosphorylation, changing its binding affinities. This active version of the androgen-AR complex, along with any additional co-factors, can then use special zinc fingers to bind to special regulatory and promoter regions of DNA, known as hormone response elements, to provoke changes in gene expression (Beato 1989, Brinkmann et al. 1999, Mangelsdorf et al. 1995). In the case of androgen-dependent systems, these hormone response elements are palindromic, and AR binds with two identical copies of the protein, that is, as a homodimer (Tsai & O'Malley 1994). Two important structural motifs in the protein's zinc fingers assist in this protein-DNA contact: an antiparallel beta sheet orients amino acid residues such that they come into full contact with DNA's phosphate backbone, and an alpha helix fits into DNA's major groove and interacts with the nucleotides themselves (reviewed in Tsai & O'Malley 1994). AR can remain bound in this transcriptionally-active stage for some time, prolonging the active life of androgens in the cell. As a direct consequence of this, very little circulating testosterone is necessary to produce large changes in gene expression (Adkins-Regan 2005).

Androgens pass through cell membranes with ease (Schlinger & Arnold 1991, Adkins-Regan 1995), so as with most other steroid hormones, there is no storage of the hormone before its release. Instead, androgens are secreted into the bloodstream as they are manufactured (Adkins-Regan 2005), and they permeate cells all through the body, not all of which need testosterone to modulate their function. This means that a tissue's sensitivity to testosterone (or lack thereof) is critical— all cells are exposed to androgens to some degree, but only cells with

AR can respond to it. When androgenic cells secrete testosterone, it acts on both the brain regions that influence behavior and on the peripheral muscle regions that exert those behaviors. Both the brain and these muscles contain AR in their cells, varying amounts of which may explain differences in how these tissues respond to testosterone and other androgens.

Androgen-driven changes in gene expression have a variety of effects on behavior (reviewed in Adkins-Regan 2005). These responses can produce changes detectable both in the short term (i.e., a few hours) and over the lifetime of the animal: in other words, androgens have both activational and developmental effects. AR can alter enzyme levels in cells, facilitate the production of neurotransmitters, and alter the thresholds necessary to produce behavior, all while stimulating cell growth and development that will alter future response to AR. Not all of these effects are desirable simultaneously or on a constant basis, so there is a need to vary circulating androgen levels, AR, or both.

Animals with mating seasons, for example, may need a baseline level of testosterone throughout their life cycle to regulate development and maintenance of sex characteristics, but vary circulating testosterone month-to-month to minimize the costs of testosterone during the non-mating season (Wingfield et al. 1990, Dufour et al. 1984). High levels of testosterone enact a high physiological cost on the individual, including suppression of the immune system (Grossman 1984, 1985) and an increase of vulnerability to parasitic infection (Saino et al. 1995). Having a higher sensitivity to testosterone in reproductively active tissues means less testosterone is necessary to produce the required effects, potentially minimizing the cost of high levels of testosterone on the immune system and other tissues.

There is extensive variation in how much AR is expressed in cells (e.g., Holmes & Wade 2005, Neal & Wade 2007). This leads to variation both in how sensitive a particular muscle or

brain region is to androgens, across both individuals and species. As a result, it is likely that peripheral AR expression could be used to regulate behavioral responses (Holmes & Wade 2005). Thus, when androgen-associated behaviors are necessary or advantageous, as they are in the breeding season or in populations with a high degree of male-male competition, these behaviors could be facilitated by increased levels of circulating androgens, higher sensitivity to testosterone and other androgens, or an interaction of the two.

**Androgens and Behavior in Anole Lizards**

Lizards in the *Anolis* genus, known commonly as anoles, are an excellent group in which to study the association between androgens and behavior. A remarkably high diversity of species makes the genus well-suited for comparative work: of the approximately 400 species in the genus, around forty species exist on the island of Hispaniola alone (Schwartz & Henderson 1991). In addition, there is an extensive body of literature on anole behavioral neuroendocrinology, most of which has focused on the green anole, *Anolis carolinensis* (reviewed in Wade 2011). Further, anole behavior is easily quantifiable through field observations – both copulatory and communicatory behaviors are highly visible (e.g., Greenberg 1977, Johnson & Wade 2010).

Anole displays primarily consist of extension of a colorful throat fan called a dewlap, in combination with push-up and headbob displays. These displays facilitate a wide variety of social interactions, including territorial defense and courtship (Jenssen 1977). Although both males and females have dewlaps, the male dewlap is larger and is used more frequently (Jenssen et al. 2000). Temporal patterns of dewlap display are species-specific, as each species exhibits stereotypical rates and duration of dewlap extension (Jenssen 1977, Johnson & Wade 2010).

Additionally, across anole species, there is a huge diversity of dewlap size, color, and pattern, differences that may be involved in species recognition (Williams & Rand 1977, Nicholson et al. 2007).

Movement of the dewlap is controlled by the ceratobranchial and ceratohyal cartilages and the ceratohyoid (CH) muscles on both sides of the throat. Contraction of the CH muscles, which are attached on either side of the throat to the ceratohyal and first ceratobranchial cartilages, exert a lever-like action on the second ceratobranchial cartilages, extending the dewlap (Font & Rome 1990, Bels 1990, Wade 2005). Dewlap display behaviors, like most social displays, are androgen-dependent (Crews 1978, Adkins & Schlesinger 1979, Winkler & Wade 1998, Holmes & Wade 2005). Display behaviors decrease dramatically upon castration, but they re-appear in full with exogenous administration of testosterone (Crews 1974, Mason & Adkins 1976, Crews et al. 1978). In the wild, testicular size is significantly correlated with testosterone levels, and both are greatest during the breeding season, when anoles have a greater need of mating and territorial displays (Tokarz et al. 2015).

Anole copulation behaviors are similarly androgen-dependent (Crews 1974, Mason & Adkins 1976, Crews et al. 1978), and quantifiable in the field. When anoles copulate, they evert one of two retractile intromittent organs, or hemipenes (Crews 1978). This eversion is driven by the contraction of a thin muscle that surrounds each hemipenis, called the transversus penis. After copulation, retraction of the hemipenis is driven by a muscle that runs lengthwise down the tail and attaches to the base of each hemipenis, the retractor penis magnus (RPM; Ruiz & Wade 2002). Both of these muscles are used once per copulation attempt, and express AR in their cytoplasm and nuclei.

The direct involvement of testosterone in social behaviors led researchers to predict that interspecific variation in anole behavior in the wild would be associated with variation in circulating androgen levels. However, Husak & Lovern (2014) found that in Caribbean anoles, ecomorphs (i.e., groups of species with similar ecology and morphology) with higher rates of both display behavior (measured as the total proportion of time spent displaying during a focal field observation) and aggression (measured through the response to intruder trials) were not necessarily those with the higher amounts of circulating testosterone. These results indicate that testosterone alone is not enough to explain behavioral differences. Instead, differences among anole species in sensitivity to testosterone, that is, differences in AR, may be more important in regulating behaviors.

Previous studies that have measured AR expression in anoles have only investigated a single species, *Anolis carolinensis*, the Carolina green anole. While all of these have measured AR expression through immunofluorescent imaging, Rosen et al. (2002) and Neal & Wade (2007) also measured AR via in-situ mRNA hybridization. Rosen et al. (2002) found AR expression in the regions of the anole brain associated with reproductive behavior, with females in that study (which used tissues from the non-breeding season) exhibiting slightly more AR expression than males. Additionally, this study found AR in the cytoplasm, consistent with findings in other vertebrate species (Rosen et al. 2002). Holmes & Wade (2005) found AR expression in the CH and RPM muscles, and furthermore, found no difference in AR expression between individuals from the breeding season and non-breeding season. When lizards were treated with testosterone, AR expression was increased in the RPM, but not in the CH (Holmes & Wade 2005). Neal & Wade (2007) found that AR expression in the kidneys (which function as accessory glands in reptiles, producing the non-sperm components of the ejaculate (Johnson et

al. 2011)) was a better predictor of social behavior than circulating testosterone, and found almost no differences in AR expression between high-displaying and low-displaying individuals; this study also found no correlation between AR expression in the CH or the RPM and their behavioral use.

Although Neal & Wade (2007) did not find a clear relationship between AR expression in these tissues and their associated behaviors, it is possible that AR expression could be responsible for differences in behavior *among* species. To test this hypothesis, I measured AR expression and behavior across multiple species of *Anolis*.

**Anole Ecomorphology**

On the islands of the Greater Antilles (Cuba, Hispaniola, Jamaica, and Puerto Rico), anole lizards have repeatedly, independently evolved into sets of microhabitat specialists, or ecomorphs (Losos et al. 1998). Six anole ecomorphs exist, although not all species of anoles fit neatly into these categories. Ecomorphs are distinguished by convergent morphologies that have evolved in tandem with their ecology, and named after the specific niche they use: crown giant, twig, trunk-crown, trunk, trunk-ground, and grass-bush (Williams 1972, 1983). Within each ecomorph, distantly-related species exhibit remarkable similarities in their dorsal color, body size, limb proportions, and behavior (reviewed in Losos 2009). Lizards adapted to the same microhabitats exploit them in similar ways, and both foraging and predator deterrence behaviors are broadly consistent within ecomorphs (Williams 1983, Johnson et al. 2008).

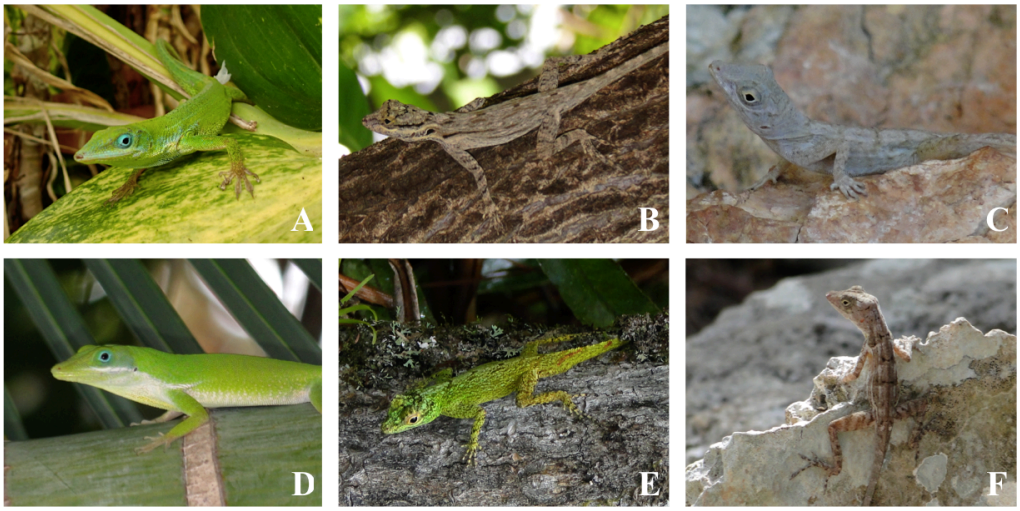


**Figure 1.** Three pairs of closely-related anole species from the Dominican Republic. Trunk-crown anoles (A: *Anolis chlorocyanus*, D: *Anolis coelestinus*), trunk anoles (B: *Anolis brevirostris*, E: *Anolis distichus*), and trunk-ground anoles (C: *Anolis cybotes*, F: *Anolis longitibialis*). Photos by M. Johnson and A. Kahrl (*A. chlorocyanus*).

In this thesis, I studied the behavior of adult male lizards in three pairs of closely related species (Figures 1 and 2), in the Dominican Republic: two species in the trunk-crown ecomorph (*Anolis chlorocyanus,* and *A. coelestinus,* two species of Hispaniolan green anole), two in the trunk ecomorph (*A. brevirostris,* the short-nosed anole, and *A. distichus,* the bark anole), and two in the trunk-ground ecomorph (*A. cybotes*, the large-headed anole and *A. longitibialis*, the Isla Beata anole).

The two trunk-crown species, *Anolis chlorocyanus* and *A. coelestinus*, are medium-large (the average snout-to-vent (SVL) length of adults is 69 and 64 mm, respectively), green arboreal lizards, which occupy the trunk and branches of trees. *Anolis chlorocyanus* is found throughout the island at moderate temperatures, preferring mixture of sunny and shaded environments. Its counterpart *A. coelestinus* is restricted to southern Haiti and the southwest coast of the

Dominican Republic, can tolerate dryer environments, and prefers cool, shaded perches (Schwartz & Henderson 1991).

The two trunk species, *Anolis brevirostris* and *A. distichus,* are relatively small (average adult male SVL 44 and 51 mm, respectively), mottled grey or brown lizards that primarily occupy the trunk and lower branches of trees. *Anolis brevirostris* is restricted to the two main mountain ranges of Hispaniola and the coast immediately south of these, and prefers larger trees, but can also be found on fence posts and in a variety of sunny and shady environments. *Anolis brevirostris* can tolerate dryer environments than *A. distichus*, which is found in habitats across Hispaniola, but prefers shade to sunlight. *Anolis distichus* has adapted to life in a variety of environments, including forest edges, fields, and even shrubs more characteristic of grass-bush anoles (Schwartz & Henderson 1991).

*Anolis cybotes* and *A. longitibialis* are gray or brown trunk-ground lizards, around the same size of their trunk-crown counterparts (average adult male SVL 67 and 64 mm, respectively) but occupying only the lower part of tree trunks and the surrounding rocks and terrain. *Anolis cybotes* often perches on the lower half of tree trunks, facing down towards the ground with its head bent at an angle for long periods of time. *Anolis longitibialis*, on the other hand, is restricted to a small section of the southern tip of the island and adapted to much dryer environments, often small caves and crevices (Schwartz & Henderson 1991).

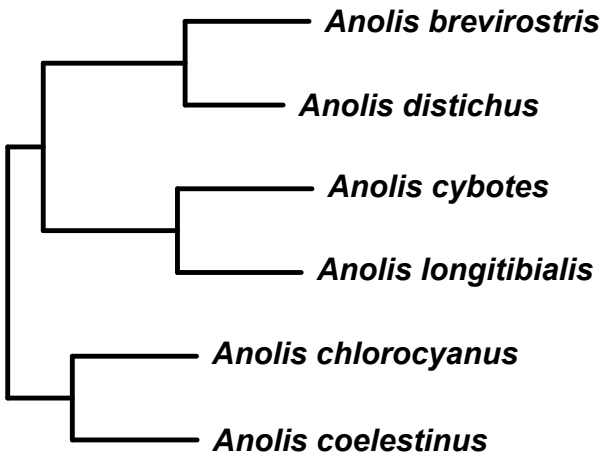


**Figure 2.** Phylogeny of the six focal species, adapted from Pyron et al. (2013).

## Hypotheses

Using these six species in three ecomorphs, I tested the hypothesis that species with higher rates of dewlap and copulation behavior will have higher AR expression in the associated muscles. More specifically, I predicted that in my six focal species, (1) lizard species with higher density of AR in their CH will have higher rates of dewlap use, and (2) lizard species with higher density of AR in their RPM will have higher rates of copulation. If these predictions are supported, then (3) AR expression in one muscle should not predict AR expression in the other, as AR would be associated with differences in behavior unique to each muscle. Finally, I predicted that (4) within each ecomorph pair of species, species with a higher rate of behavior would have higher AR density in the associated muscles.

# Materials and Methods

## Field Methods

We studied the six species of anoles in the Dominican Republic, during June 2006, 2011, and 2015, in the following locations: *Anolis coelestinus*, *A. brevirostris*, and *A. cybotes* were studied on the grounds of the Coralsol Beach Resort in Barahona (18.062, -71.111), *Anolis distichus* was studied south of Baní (18.232, -70.347), *Anolis chlorocyanus* was studied near the town of Ocoa (18.525, -70.510), and *Anolis longitibialis* was studied in the town of Manuel Goya (17.836, -71.450).

For each species, we performed 10-120 min focal behavioral observations on adult males, for a minimum of 20 h per species (Table 1). Animals were located for observations by walking slowly through the field sites, until locating an undisturbed lizard. We visually determined the sex of the lizards by observing relative body and head size (adult males are generally larger than females, with larger heads), and identifying the presence of a large dewlap and/or a bulge behind the tail (which indicates the presence of hemipenes). As male anoles are territorial and generally remain in a small home range (Rand 1967, Decourcy & Jenssen 1994), we avoided repeating observations in the same immediate area to minimize the probability of watching the same lizard more than once. During observations, we recorded the frequency of dewlap extensions, and used a digital stopwatch to measure the total amount of time that a lizard's dewlap was extended. For each individual, we calculated dewlap frequency as dewlap extensions per minute, and dewlap duration was calculated as the average time span for a single dewlap extension (in seconds). Additionally, we recorded each time an individual was observed copulating, and calculated the average copulation rate for each species for use in subsequent analyses.

After observations were completed, we captured ten adult males per species by hand or noose, temporarily housed them in small plastic containers for transport, and transported them to Trinity University via cargo shipping. All procedures were approved by Trinity's Animal Research Committee (protocols NSF_050213_MAJ3, 011415_MJ1, and 042811-MJ1) and by the Ministerio de Medio Ambiente y Recursos Naturales (Environment and Natural Resource Ministry) in the Dominican Republic.

**Table 1**. Behavioral data collected for six focal species

| Species | Total Observation Time (h) | Number of Lizards Observed | Average Observation per Lizard (min) | Ecomorph |
|---|---|---|---|---|
| *Anolis chlorocyanus* | 23.3 | 22 | 63.5 | Trunk-Crown |
| *Anolis coelestinus* | 60.0 | 87 | 41.4 | Trunk-Crown |
| *Anolis brevirostris* | 57.5 | 85 | 40.6 | Trunk |
| *Anolis distichus* | 30.8 | 37 | 49.9 | Trunk |
| *Anolis cybotes* | 74.1 | 113 | 39.3 | Trunk-Ground |
| *Anolis longitibialis* | 33.6 | 31 | 65.0 | Trunk-Ground |

**Muscle Tissue Collection**

Lizards were euthanized by rapid decapitation within five days of capture. I then dissected jaw and tail tissues, which were immediately flash-frozen on dry ice. Tissues were stored at -80 ºC until further processing.

I cryosectioned jaw and tail tissues at 20 µm in six alternate series. I thaw-mounted tissues on SuperFrost Plus microscope slides (Fisher Scientific; Hampton, NH) and stored at -80 ºC. I then stained one series per tissue for each individual with hematoxylin and eosin (H&E) to locate the muscles of interest for immunofluorescence.

**Protein Labeling by Immunofluorescence**

To quantify AR expression in muscle tissues, I performed immunofluorescence staining using a second series of cryosectioned tissue. Samples from all individuals were included in a single run for each tissue type. After allowing the slides to defrost for 10 min, tissues were fixed in 4% paraformaldehyde (PFA) for 10 min, rinsed three times in phosphate-buffered saline (PBS), and encircled on the microscope slide with a pap pen. Tissues were blocked for 2 h at room temperature in 200 µL blocking solution (4% normal goat serum and 0.3% Triton-X detergent in PBS), during which time they were covered with parafilm and placed in an airtight, plastic container to maintain humidity levels. Then, slides were incubated with primary antibody (PG-21 rabbit anti-AR polyclonal antibody (EMD Millipore)), at a concentration of 1:500 for tails and 1:250 for jaws (in 2% normal goat serum and 0.3% Triton-X in PBS), in airtight containers for approximately 48 h at 4 ºC. After the primary incubation period, tissues were rinsed three times in PBS and incubated in secondary antibody solution (1:1000 AlexaFluor 594-conjugated goat anti-rabbit antibody and 0.3% Triton-X in PBS), in total darkness for 2 h at room temperature. Finally, slides were coverslipped with DAPI Fluoromount-G and stored flat in a light-proof container to dry.

In each run, I included a no-primary control to quantitatively determine baseline levels of background staining. This control consisted of at least two slides with *Anolis carolinensis* (a species whose AR expression in jaw and tail muscles has been previously characterized using PG-21; Holmes & Wade 2005, Neal & Wade 2007) jaw or tail tissue and was treated exactly like every other slide, but was incubated without primary antibody. I also conducted a pre-adsorption control by staining one tail and one jaw tissue from each of the species as per the usual protocol, but with primary antibody that had previously been incubated with 20x molar mass of purified

AR protein (custom peptide from Biosynthesis, Inc., Lewisville, TX). PG-21 is raised against the first 21 amino acids of the human AR protein, and in the binding domain, anole AR has 97% sequence identity with human AR.

**Image Capture**

After the slides dried for a minimum of 1 day, I imaged slides at 400X magnification on a Nikon A1 Confocal microscope (Nikon Instruments). The DAPI laser and TRITC laser parameters were optimized to detect AlexaFluor 594 as follows. Lasers were fired in a channel series for capture, and the final image was captured using the full field of view at 2048 x 2048 pixels. Line averaging (4x) was used to reduce background signal. Capture settings were standardized by adjusting the laser power, gain, and offset to use the full width of the histogram for both DAPI and AlexaFluor 594 on a single slide from the run, chosen from the tissues with the highest AR expression, such that the signal on slides always falls within the device's dynamic range limitations. The pinhole was always set to 1.2, as determined by the airy unit (AU) for the longest wavelength (594 nm).

In the CH, I measured nuclei in two 320 μm x 320 μm regions on one side of the animal (for a total of 204,800 $\mu m^2$), near the rostrocaudal center of the muscle (following Neal and Wade 2007). In the RPM, I measured nuclei in one 320 μm x 320 μm regions on one side of the animal (for a total of 102,400 $\mu m^2$), near the rostrocaudal center of the muscle (following Neal and Wade 2007).

**Image Processing**

I conducted image processing in the FIJI program (Schindelin et al. 2012). After setting a threshold to eliminate the dark background from images in the DAPI channel, such that only nuclei are visible to the software, I selected all nuclei in each image with the Analyze Particles function. Using the AlexaFluor594 channel (which captures fluorescence from the fluorophores-conjugated secondary antibody, AlexaFluor 594, and thus AR), I used a median filter to reduce noise and measured mean brightness and integrated density for each individual nucleus. I wrote a macro script to automate this process for all the images I captured, quantifying AR expression in the nuclei and exporting nucleus integrated density values for each individual into Microsoft Excel.

Protein expression in nuclei can be measured through its integrated density, which is roughly equivalent to the area of a nucleus multiplied by its average brightness. Although both brightness and integrated density are proportional to the amount of fluorescent-labeled protein, the measure of average brightness can only represent the average nucleus. For example, in the hypothetical case of two individuals with identical average nuclei brightness scores, if one individual has larger nuclei than the other, then that individual needs to have a correspondingly larger amount of AR being expressed in its tissues. This would therefore correspond to a greater sensitivity to testosterone than the individual with smaller nuclei, a nuance that a simple measure of average brightness would fail to convey.

**Statistical Analysis**

I conducted all statistical analyses in R 3.3.1 (R Core Team 2016) and IBM SPSS Statistics for Windows 24.0 (IBM Corporation 2016). I first tested for differences in AR

expression and behavior using ANOVAs in SPSS, following with Tukey's HSD as a post-hoc test. I tested for correlations between AR density (as measured by both species average nucleus brightness and species average nucleus integrated density) and behavior (as measured by the species average number of dewlap extensions per minute and copulation rate per hour), using generalized least squares correlation assuming Brownian evolution of traits, using the *gls* function in *nlme* (Pinheiro et al. 2009), hence referred to as phylogenetic correlations. I then conducted t-tests to determine differences in AR expression within each ecomorph pair. Finally, I calculated Blomberg's K and Pagel's lambda as tests for phylogenetic signal (Pagel 1999, Blomberg et al. 2003) using the *phylosig* function in *phytools* (Revell 2012).

# Results

## Dewlap Display and Copulation Behavior

Dewlap display and copulation behaviors differ dramatically among the six species in this study (Table 2). Species differed in dewlap rate (ANOVA: $F_{5,213} = 17.48$, $p < 0.001$) and duration of dewlap extension (ANOVA: $F_{5,160} = 8.54$, $p < 0.001$), as follows. The two trunk-crown species (*A. chlorocyanus* and *A. coelestinus*) had among the longest durations of dewlap extension. The two trunk anole species (*A. distichus* and *A. brevirostris*) dewlapped most frequently, although for the shortest duration. The two trunk-ground species differed dramatically in their rate of dewlap extension, with *A. cybotes* doing so six times more often than *A. longitibialis*, on average. Each ecomorph pair had a higher-displaying species that displayed more frequently for a shorter time, and a low-displaying species, that conversely extended their dewlaps less frequently, for longer durations. *Anolis chlorocyanus* was observed copulating the most often (0.215 copulations per hour). *Anolis longitibialis* was not observed copulating in 33.6 hours of focal observations.

**Table 2**. Behavioral data for six focal species

| Species | Average Dewlap Rate, ± 1 SE (extensions / minute) | Average Dewlap Duration, ± 1 SE (seconds) | Observed Copulation Rate (copulations / hour) | Ecomorph |
|---|---|---|---|---|
| *Anolis chlorocyanus* | 0.047 ± 0.115 | 27.554± 4.263 | 0.215 | Trunk-Crown |
| *Anolis coelestinus* | 0.125 ± 0.082 | 9.323 ± 3.178 | 0.034 | Trunk-Crown |
| *Anolis brevirostris* | 0.513 ± 0.082 | 2.155 ± 2.714 | 0.051 | Trunk |
| *Anolis distichus* | 1.023 ± 0.089 | 1.586 ± 3.066 | 0.065 | Trunk |
| *Anolis cybotes* | 0.226 ± 0.082 | 7.790 ± 2.644 | 0.079 | Trunk-Ground |
| *Anolis longitibialis* | 0.038 ± 0.097 | 21.701 ± 3.788 | 0.000 | Trunk-Ground |

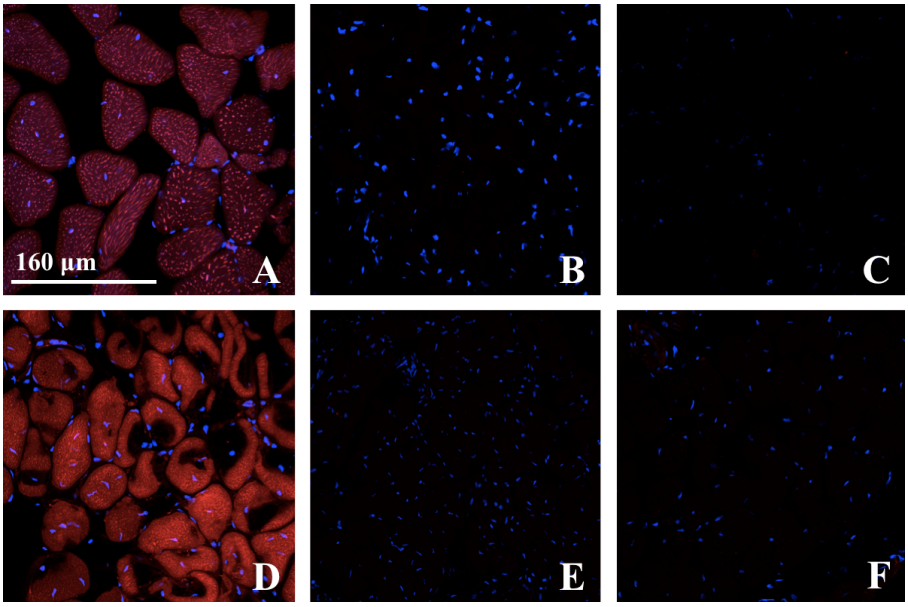


**Figure 3**. Immunofluorescent staining for AR protein shows signal in both the CH (A) and RPM (D). No-primary controls show minimal background staining for both the CH (B) and the RPM (E). Protein pre-adsorption controls show minimal background staining for both the CH (C) and RPM (F).

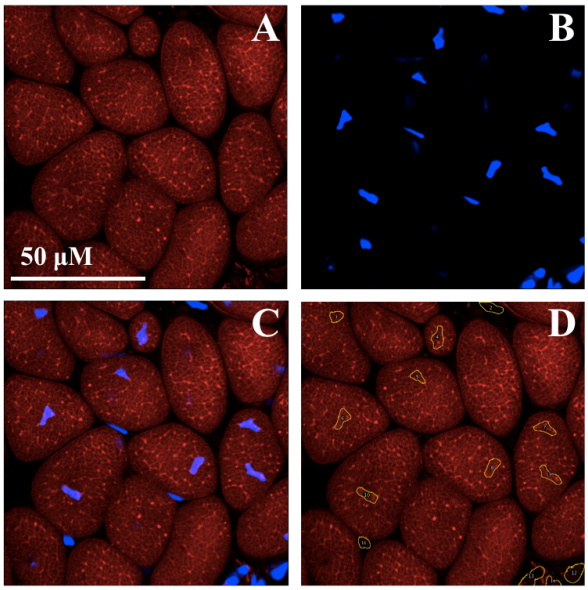


**Figure 4.** Cropped image of RPM shows the progression of software labeling of nuclei in ImageJ. Data from the AR channel (A) are matched with data from the DAPI channel (B) to produce a composite (C), from which nuclei can be selected and measured (D).

**Interspecific Variation in AR Expression**

Immunofluorescent labeling was specific to AR in both muscles (Figure 3), and the ImageJ macro correctly identified nuclei from the DAPI channel (Figure 4).

AR expression in the CH muscle did not significantly differ among the six species ($F_{5,50}$ = 0.59, $p$ = 0.071; Figure 5). Among each species, there was substantial variation in integrated density measures, suggesting high variability in AR expression in this muscle. Across species, there is a trend towards a relationship between the rate of dewlap extension and AR expression in the CH, such that species with higher rates of dewlap extension have marginally higher average integrated density in the cell nuclei of the CH (phylogenetic correlation, $t_4$ = 2.33, $p$ = 0.081; Figure 6).

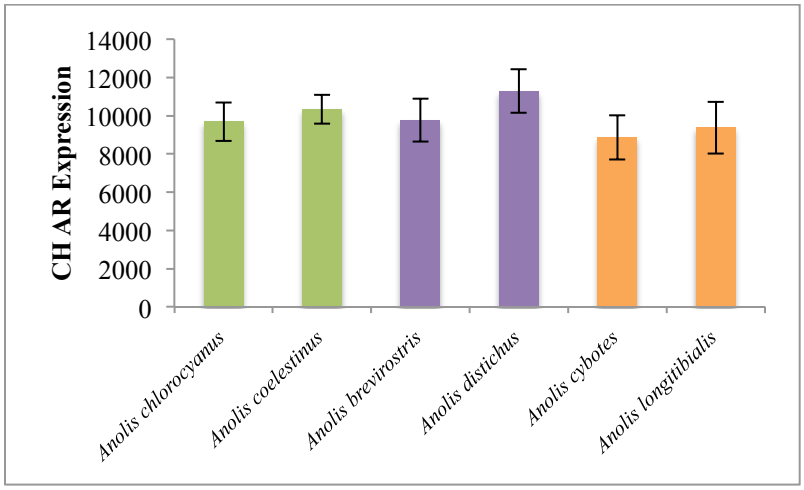


**Figure 5.** Ceratohyoid AR expression for six *Anolis* species, measured as average nucleus integrated density. Error bars are ± 1 standard error from the mean. Trunk-crown species are in green, trunk species in purple, and trunk-ground species in orange.

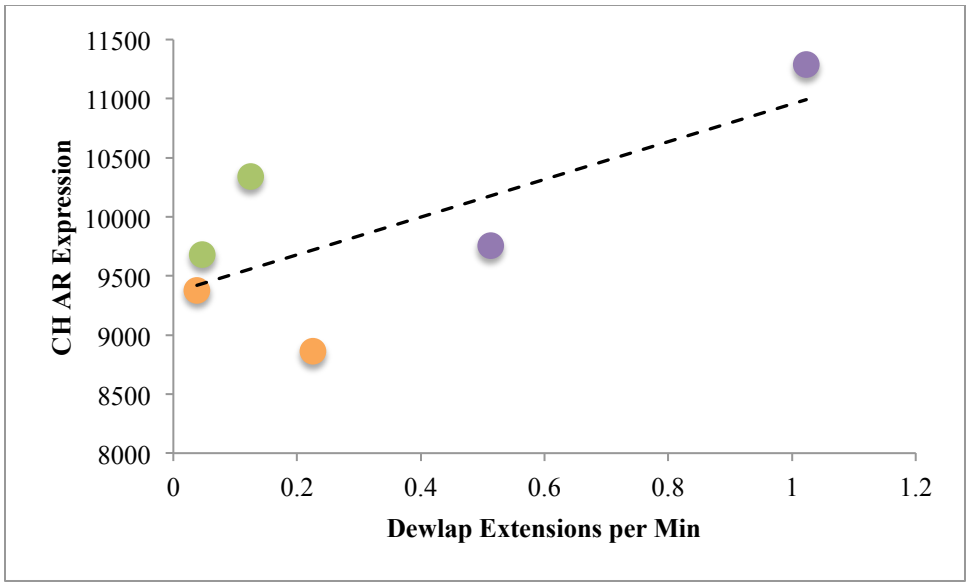


**Figure 6.** Species with higher average rates of dewlap extensions have marginally more AR expression in the ceratohyoid, the muscle that controls this extension. AR expression is quantified as the average nucleus integrated density. Trunk-crown species are in green, trunk species in purple, and trunk-ground species in orange.

The six species differ in their AR expression in the RPM ($F_{5,50} = 3.10$, $p = 0.016$; Figure 7). Only *A. brevirostris* and *A. chlorocyanus*, and *A. brevirostris* and *A. longitibialis* differ significantly from each other; *A. brevirostris* had the highest AR expression in the RPM, while *A. chlorocyanus* and *A. longitibialis* both had the smallest. As with the CH, there was substantial variation in integrated density measures, suggesting high variability in AR expression in this muscle. No relationship is evident between the rate of copulation and AR expression in the RPM (phylogenetic correlation, $t_4 = -0.17$, $p = 0.88$; Figure 8).

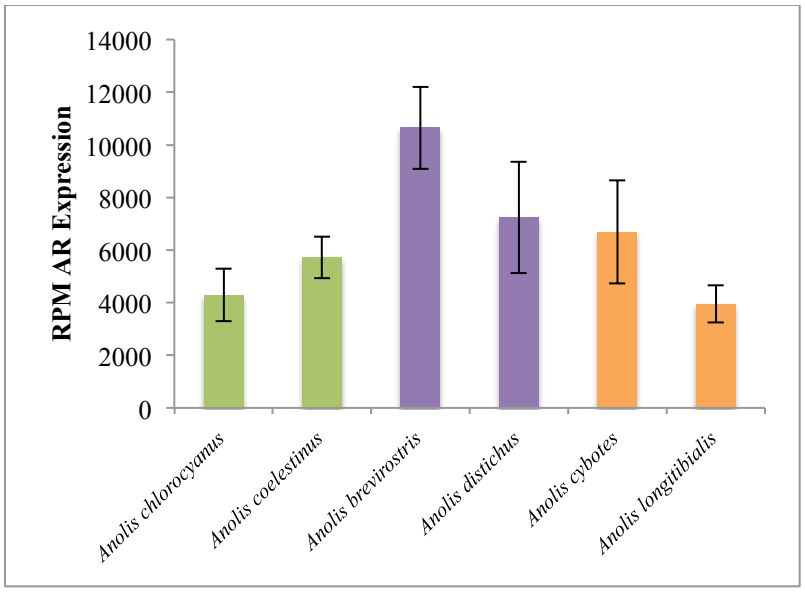


**Figure 7.** Retractor penis magnus AR expression for six *Anolis* species, measured as average nucleus integrated density. Error bars are ± 1 standard error from the mean. Trunk-crown species are in green, trunk species in purple, and trunk-ground species in orange.

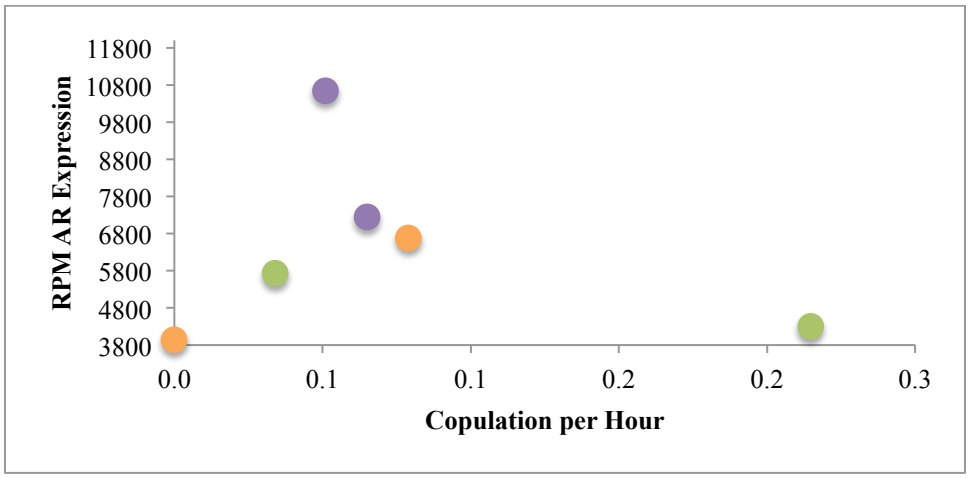


**Figure 8.** There was no relationship between the observed copulation rate and AR expression in the RPM, the muscle that controls hemipenis retraction. AR expression is quantified as the average nucleus integrated density. Trunk-crown species are in green, trunk species in purple, and trunk-ground species in orange.

I found no relationship between AR expression in the CH and AR expression in the RPM (phylogenetic correlation, $t_4$ = -0.63, $p$ = 0.56; Figure 9). I found no phylogenetic signal for AR expression in either the CH (K = 0.81, $p$ = 0.32; λ = 6.6e-05, $p$ = 0.71) or the RPM (K = 0.98, $p$ = 0.19 ; λ = 0.423, $p$ = 1).

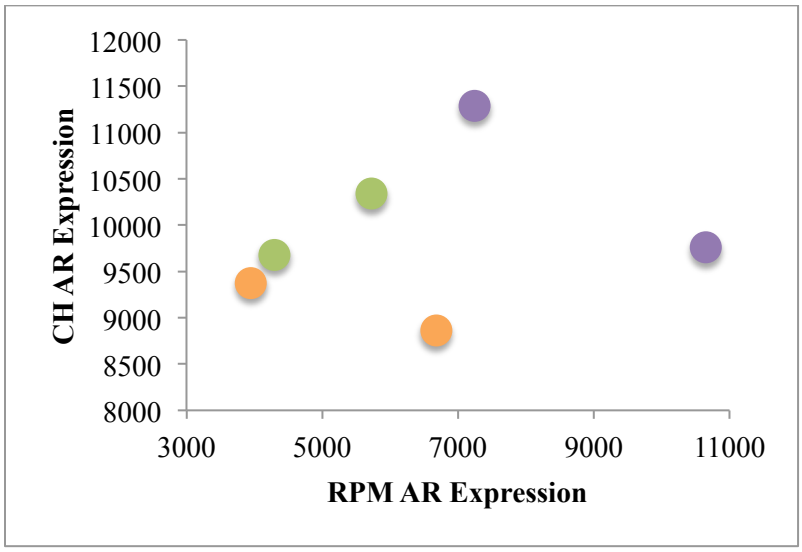


**Figure 9.** AR expression in the CH is not associated with AR expression in the RPM.

## Variation in AR with Ecomorph Pairs

When comparing the two species in each ecomorph pair, I found no difference in CH AR expression (Figure 5) between the two species in either the trunk-crown ecomorph ($t_{15}$ = -0.52, $p$ = 0.61), the trunk ecomorph ($t_{16}$ = 0.94, $p$ = 0.36), or the trunk-ground ecomorph ($t_{19}$ = -0.29, $p$ = 0.77). Likewise, in the RPM (Figure 7), AR did not differ between species pairs in either the trunk-crown ecomorph ($t_{18}$ = -1.08, $p$ = 0.30), the trunk ecomorph ($t_{17}$ = -1.31, $p$ = 0.21), or the trunk-ground ecomorph ($t_{15}$ = -1.37, $p$ = 0.19).

## Discussion

The first prediction, that AR expression in the CH would correlate with the rate of dewlap extension, was partially supported by the data (Figure 6). The high degree of variability in my results (individuals' average nucleus integrated density values ranged from less than 4,000 to almost 20,000) means there was no detectable difference in AR expression in this muscle among species, limiting interpretation of the data (Figure 4). Because the phylogenetic analyses used in this study only analyze species means, this variation is not considered in the evolutionary analysis. Yet, the results from the phylogenetic correlation are in the direction I predicted – and as more species are measured, this pattern (or its absence) will become clearer. Fuxjager et al. (2015) found a similar pattern in passerines, where species that perform more complex wing displays have more AR in the nuclei of the wing muscles.

The second prediction, that AR expression in the RPM would correlate with the observed average copulation rate, was not supported by the data. Again, the measure of AR expression in this muscle showed a large degree of variability: individual lizards' averages ranged from less than 2,000 to over 22,000. However, here there were significant differences in AR expression amongst the species, between *A. brevirostris* and *A. chlorocyanus*, and between *A. brevirostris* and *A. longitibialis*. These represent the difference between the species with the most AR expressed, *A. brevirostris*, and the two species with the least AR expressed in the RPM, *A. chlorocyanus* and *A. longitibialis* (Figure 7). The large degree of individual variation seen here could be related to differences in circulating testosterone, of which baseline levels for different species range by a factor of 4x (Husak & Lovern 2014) and which is known to influence RPM AR expression (Holmes & Wade 2005).

My third prediction, that AR expression could be controlled by different factors in different

muscles, is supported by the results of this study. AR expression in one muscle is not associated with AR expression in the other (Figure 9). Contingent on the addition of more species in future studies, it is possible that the AR in the CH could be tied to display behavior, as in Fuxjager et al. (2015), and that AR in the RPM could be constrained by anoline evolutionary history or other factors. Holmes & Wade (2005) found that testosterone increased AR density in the RPM, but not the CH, in *A. carolinensis*. On average for this study, the proportion of standard error to mean AR expression is 0.11 in the CH and 0.21 in the RPM (Figure 10). For Holmes & Wade (2005), those proportions are 13.3% and 19.2%, respectively; although the former study examined only a single species, both the relative magnitudes of error between muscles and the approximate values of these proportions are similar across studies. Standard error is dependent on sample size, but Holmes & Wade reports only 8 individuals for the breeding season, comparable to the 10 individuals per species in my own study.

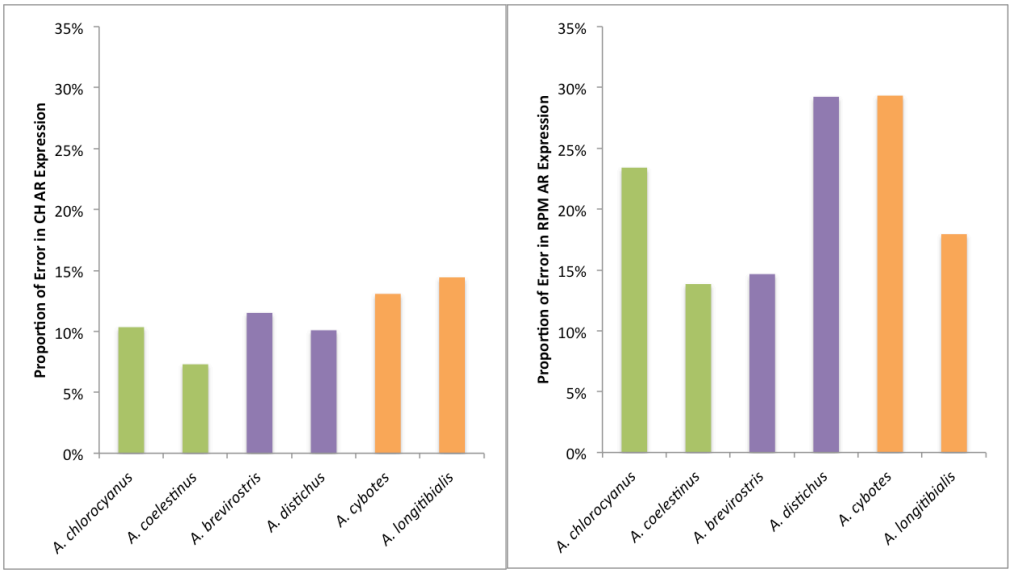


**Figure 10.** There is a greater proportion of error to the mean in the RPM (right) than the CH (left), and this proportion varies between species.

Husak et al. (2007) reports similar degrees of variation in circulating testosterone levels for *A. carolinensis*, with the proportion of standard error to mean circulating testosterone 0.17 on average. If circulating testosterone directly affects AR in the RPM, this could explain why I found a higher degree of variation for AR expression in the RPM than in the CH. To estimate a potential influence of circulating testosterone on AR expression in both muscles, I extracted approximate values for circulating testosterone from Husak & Lovern (2014) for four of my species: *A. brevirostris, A. coelestinus, A. cybotes*, and *A. distichus*. In both muscles, circulating testosterone seems to predict AR expression. However, this relationship is potentially stronger with the CH (Figure 11) than the RPM (Figure 12).

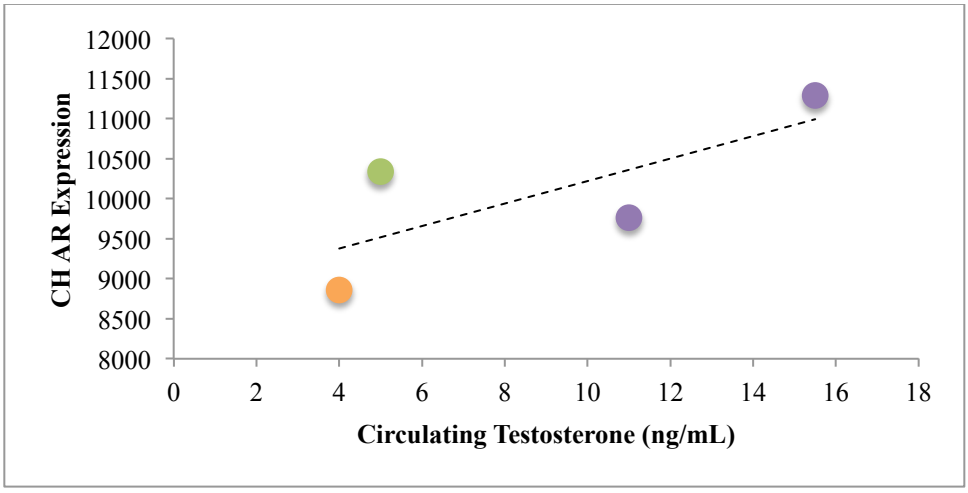


**Figure 11.** Circulating testosterone (data from Husak & Lovern 2014) may predict AR expression in the ceratohyoid ($R^2 = 0.553$). The dashed line is a best fit as approximated by a linear model, $y = 140x + 8815$.

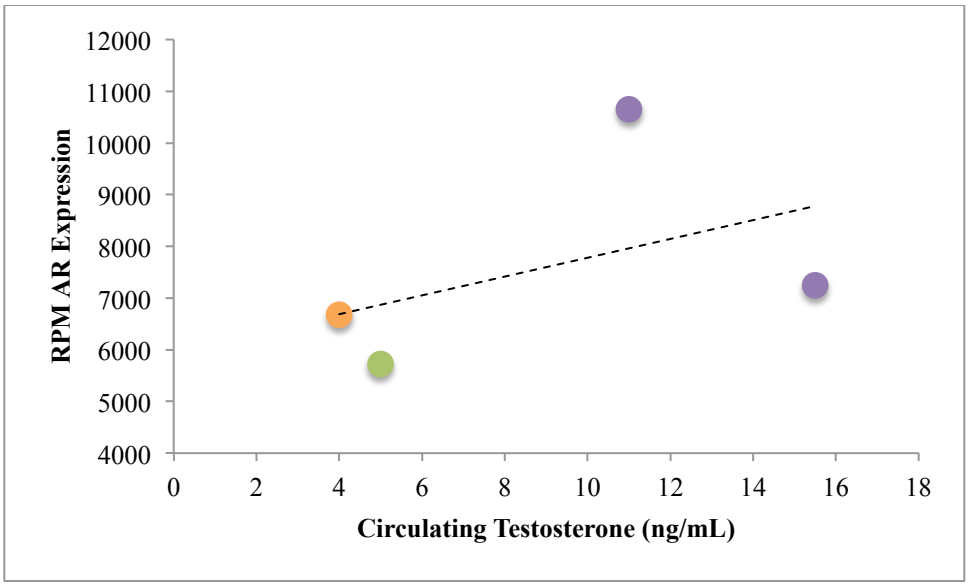


**Figure 12.** Circulating testosterone (data from Husak & Lovern 2014) may predict AR expression in the retractor penis magnus ($R^2$ = 0.209). The dashed line is a best fit as approximated by a linear model, $y = 182x + 5961$.

I found little support for my fourth prediction, that within ecomorph pairs, behavior would be positively correlated with AR expression in the associated muscle. In the CH, both trunk and trunk-crown anole pairs vary in the expected directions (Figure 3). However, the trunk-ground species pair does not. The high degree of variation in AR expression within species may hide a possible relationship with behavior within ecomorph pairs, but there is as of now little support for this prediction.

I found a large amount of extra-nuclear AR in both the CH and RPM. This is consistent with previous findings in the brain of a single species, *A. carolinensis* (Rosen et al. 2002). This free AR could work as a reserve, meaning only a small amount of testosterone is necessary to produce a large effect in the cell. If the level of AR is high enough, a threshold at which there is more AR than circulating testosterone would mean that species differences in AR might not necessarily correlate with species differences in behavior.

It is possible the genes that regulate or influence behavior in these muscles vary in the presence of androgen response elements across species. If some species have genes with response elements that better favor AR binding (Shaffer et al. 2004), those species would have a greater influence of androgens on behavior regardless of absolute AR concentration in the muscles. Different genes could influence behavior across species, further complicating the relationship between AR and behavior.

It is important to note that the lizards we caught for this study are not necessarily the same ones I observed in the field. If the high degree of variance in AR expression is not an artifact of my data collection, it is possible that ten individuals per species are simply not enough to uncover subtle influences of AR on behavior. Future studies could look at measuring AR in lizards that have individual observational data points, thus answering this question from a different perspective. Within a single species, it is possible that individuals with higher display rates have higher AR, and that this within-species variation is much larger than across-species differences.

This study shows suggestive support for the idea that display behavior is associated with AR expression in the muscles. Fuxjager et al. (2015) demonstrated this was the case for seven species of tropical passerines: that study found both that display complexity was associated with AR in the muscles that exert these displays, and that for any given species, AR in one muscle or region did not always correlate with AR in another muscle. This is consistent with the results of this study, and provides support for AR-dependent control of display but not copulatory behavior.

AR expression, as well as behavior, may be controlled by different factors in different muscles. For the CH, that may be display behavior; for the RPM, that may be circulating

testosterone. This is the second study to date looking at the evolutionary relationship between AR expression and behavior across species, and further contributes to our understanding of the cellular mechanisms that drive social behavior in anoles. The results from this study open the door for the continued study of this relationship in anole lizards.

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