4-19-2013

THE EFFECT OF VARIATION IN ENDOCRINE MECHANISMS ON NATURAL DISPLAY BEHAVIOR IN CARIBBEAN ANOLIS LIZARDS

Diego Castro
Trinity University

THE EFFECT OF VARIATION IN ENDOCRINE MECHANISMS ON NATURAL DISPLAY BEHAVIOR IN CARIBBEAN *ANOLIS* LIZARDS

DIEGO CASTRO

A DEPARTMENT HONORS THESIS SUBMITTED TO THE
DEPARTMENT OF BIOLOGY AT TRINITY UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR GRADUATION WITH DEPARTMENTAL HONORS

DATE _____

_____ THESIS ADVISOR

_____ DEPARTMENT CHAIR

_____
ASSOCIATE VICE PRESIDENT FOR ACADEMIC AFFAIRS,
CURRICULUM AND STUDENT ISSUES

ABSTRACT

Sexual display behaviors often consist of elaborate performances designed to attract potential mates, and increases in circulating androgens are frequently associated with increases in sexual display behavior. In *Anolis* lizard species, display behaviors consist of dewlap (i.e., throat fan) extensions and pushups, and species can vary dramatically in their patterns of display. My objective in this study was to determine whether interspecific differences in androgen receptors in the muscles controlling dewlap extension and pushup behaviors are associated with the frequency of use of those muscles during displays. I used behavioral data for adult males of five species of *Anolis* lizards from the Barahona region in southwestern Dominican Republic. I found that there is substantial variation across species in the number of pushups and dewlaps done in their displays. I also carried out controlled arena trials, where males of the same species were put together in a small cage to provoke displays at each other, and found display patterns consistent with their natural behavior. I determined the expression of androgen receptors in the muscles through immunocytochemistry, and found the expression of androgen receptor in dewlap-controlling muscles to be associated with dewlap display behavior. In addition, I determined the muscle fiber size and found bicep muscle fiber size to be associated with pushup display frequency. This study will contribute to our understanding of the morphological basis for behavior, particularly how endocrine mechanisms can lead to variation in social display behavior.

ACKNOWLEDGEMENTS

I owe many thanks to Dr. Michele Johnson for her support, encouragement, and guidance. I have gained invaluable academic, professional and personal skills in my three years working with her, and none of this would have been possible without her help. I would like to thank Bonnie Kircher and Chris Robinson for their help in the lab—I would probably still be collecting data if it wasn't for them. I would also like to thank Ellee Cook, Thom Sanger and Natalie Jacewicz for their help and support in collecting field data in the Dominican Republic. I thank Dr. Jonathan King and Kevin Klindworth for their assistance in lab, and Jamal Murray for his help with data collection. The Howard Hughes Medical Institute, The Explorer's Club, Mind Science Foundation, and Trinity University Biology Department provided invaluable support and funding for this project.

Ethical Note

All procedures were performed with the approval of Trinity University's Animal Research Committee and the Ministerio de Medio Ambiente y Recursos Naturales, Dominican Republic.

TABLE OF CONTENTS

INTRODUCTION

Social behavior and its underlying mechanisms

Animals often perform display behaviors to communicate with other individuals. Because social displays often consist of physical movements that reveal or emphasize a particular structure that provides information about the displaying individual, these signals are frequently used to advertise the individual's condition (Kodric-Brown & Brown, 1984). The use of these display behaviors depends on morphological structures that control them, so comparing display behaviors within and across animal taxa provides an effective way to investigate the relationship between structure and function. Display behaviors are often mediated by a multitude of different physiological mechanisms (Crews & Moore, 1986; Shelley et al., 2006), such as neural circuits and hormones. Studying the way variation in these underlying mechanisms affects an organism's behavioral displays helps us elucidate the way different morphological structures function to regulate an organism's behavior.

Among the most important factors in regulating social display behavior are sex steroid hormones. Sex steroid hormones such as androgens (which generally include testosterone and its metabolites) and their receptors within cells regulate social behavior across many different animal taxa, including fish, birds, reptiles and mammals (reviewed in Adkins-Regan, 2005). Androgens, primarily testosterone and dihydrotestosterone, are principally produced in the testes and are of particular importance for mediating social and sexual traits in males across multiple animal taxa. Furthermore, testosterone is often important in both the development of the structures underlying social behaviors and their activation in adult individuals (Lovern et al., 2004; Breedlove et al., 2002). Traits associated with increased levels of circulating androgens include aggression (Crews & Moore, 1986; Marler & Moore, 1988), male courtship of females

(Meisel & Sachs, 1994), and, in some species, physiological and morphological changes such as color shifts and increased endurance (Miles et al, 2007). For example, studies in side blotched lizards (*Uta stansburiana*) have shown that testosterone frequently increases in association with larger territories, increased courtship, more frequent copulatory and territorial behavior, as well as dominance status (Sinervo et al, 2000). Additionally, it has been found in zebra finches (*Taeniopygia guttata*) that castrated males have reduced courtship and no copulation behaviors, but both of these behaviors were restored to normal levels upon receiving testosterone treatments (Harding et al., 1983). This same pattern was observed in male quail (*Coturnix coturnix japonica*) with photically regressed testes (Adkins et al., 1980).

The interactions between androgens and the traits they influence are mediated by androgen receptor (AR) protein (McGinnis & Dreifuss, 1989), a type of nuclear receptor protein that is activated upon binding to testosterone or dihydrotestosterone in the cytoplasm, which then causes its translocation to the nucleus (Lu et al., 2006). These AR proteins are frequently located in cells composing the brain structures associated with the behavior activated by the androgen (Balthazart et al., 1992; Huddleston et al., 2007). In addition to their role in activating different brain regions, AR are also found in muscles, suggesting that the activity of these muscles might be influenced by their sensitivity to androgens (Michel & Baulieu, 1980; Herbst & Bhasin, 2004).

There has been much research investigating exactly what the activational effects of androgen are in different species, primarily mammals and birds. In general, these effects include modulating the production and release of neurotransmitters and hormones, and stimulating the production of courtship behavior (Arnold & Breedlove, 1985; Adkins et al., 1980). However, most of these studies have been conducted in individual species.

While this approach is valuable, and much of our knowledge of the mechanisms of behavior comes from single-species studies, results from single-species studies may be limited in generality, as these findings may apply only to the species under investigation. While a single species exhibiting a relationship between two or more of these factors can be informative, it is helpful to find whether these relationships hold when a group of species is analyzed. For example, the frequency of social signals in a species of electric eel was found to be associated with increased testosterone, and studies on other species in its genus found these results consistent across multiple species (Dunlap & Zakon, 1998; Dunlap et al., 1998). However, a study by Brenowitz (1997) in a group of six songbird species found a different pattern. Here, sexual dimorphism in HVc (high vocal center) and RA (robust nucleus of the archistriatum) volume across multiple species was associated with sexual dimorphism in the complexity of song repertoire in these species. However, no relationship was found between RA somal size or total number of neurons and song repertoire complexity. When comparing a group of species, relationships that seemed apparent on a more focused study can often disappear, or new relationships can be found instead. Thus, in order to understand the general relationships between androgens, their receptors, and social behavior it is important to carry out such studies across groups of species.

Social displays in anoles

Anolis lizards serve as a good model for the study of the relationship between androgens, receptors, and behavior. There are almost 400 species in the *Anolis* genus, which vary dramatically in their morphology, ecology and behavior (Losos, 2009). The social behavior for these species is highly visual and easily quantifiable in the field. In addition, there is a robust

phylogeny available (Rabosky & Glor, 2010) for these groups, which allows for the study of the evolutionary relationships between display behavior and its underlying traits in these species.

Anole display behavior consists of extensions of a colorful throat fan called a dewlap, often performed in tandem with multiple pushup movements (Greenberg & Noble, 1944; Crews, 1979, 1980). These behaviors are used for territorial defense and male courtship purposes (Jenssen, 1977) as well as for species recognition (Nicholson et al., 2007) and predator deterrence (Leal & Rodriguez Robles, 1997). Courtship displays occur primarily during the summer breeding season for these species and depend on seasonal increases in androgen for their activation (Lovern et al., 2004). Castrated males cease displaying within two weeks, with testosterone treatments successfully rescuing display behavior (Mason & Adkins, 1976; Adkins & Schlesinger, 1979; Winkler & Wade, 1998).

Anole display behaviors are primarily mediated by muscles in the forelimb and jaw. Specifically, pushup displays involve sequential contraction of the bicep and tricep muscles. Dewlap extensions involve contraction of the ceratohyoid muscle in the throat, which in turn causes the extension of the second ceratobranchial cartilage (a structure lining the dewlap which permits its full extension) and the unfolding of the dewlap skin (Crews, 1980). There is a considerable degree of variation in the frequency and duration of dewlap use among different species (Johnson et al. 2010). This in turn suggests that there may be underlying differences across species in the structures involved in controlling these displays.

Mechanisms for displays in anoles

Previous studies have extensively examined *Anolis carolinensis* (the green anole) to determine the relationship between dewlap display behavior and the sizes of associated

morphologies. The muscle fiber sizes of the ceratohyoid muscle have been found to be larger in males (who display the dewlap more frequently) than females, and larger ceratohyoid fibers are associated with higher dewlap extension rates in males of this species (Neal & Wade, 2007). Additionally, the length of the second ceratobranchial cartilage and overall muscle sizes have also been found to be higher in males than females (O'Bryant & Wade, 1999). However, similar relationships between the sizes of structures and the frequencies of their use are not seen when compared across males in a of group nine *Anolis* species. Specifically, there was no significant relationship between muscle fiber size (Johnson & Wade 2010) or muscle fiber type composition (R. Khozein et al., pers. comm.) in the ceratohyoid muscle with relation to its use in dewlap display frequencies.

Another factor that influences dewlap display rate is circulating levels of plasma testosterone. In *A. carolinensis*, increased levels of testosterone generally increase male sexual display and copulatory behaviors (Neal & Wade, 2007). When compared across a group of Caribbean anole species, however, variation in levels of circulating testosterone had no relationship with dewlap display frequencies (J. Husak & M. Lovern, pers. comm.). This suggests that there may be another factor involved in mediating the interaction between testosterone and display behaviors; while the sizes of morphological structures, muscle fiber type, and circulating testosterone have not explained interspecific differences in display behavior, no studies have yet investigated AR expression in display-related muscles across species.

In this study, I used a group of closely-related Caribbean *Anolis* lizards (anoles) to study the association of AR in muscles and the behavior that these muscles control. Specifically, I tested the hypothesis that AR expression in the muscles controlling anole display behavior is

associated with the frequency of these muscles' use and that this trend would be consistent across several species. I focused this study on five species of anoles endemic to the Barahona region of the Dominican Republic—*A. bahorucoensis* (the Bahoruco long-snouted anole), *A. brevirostris* (the shortnose anole), *A. coelestinus* (the Hispaniolan green anole), *A. cybotes* (the largehead anole), and *A. olssoni* (the desert grass anole) (Figure 1). Additionally, where available, I have included ceratohyoid muscle data and behavioral data from *A. carolinensis*, the only other anole species in which such mechanistic studies of display behavior have been performed. These six species show significant variation in their dewlap extension and pushup rates (Johnson 2007; Johnson & Wade, 2010). I predicted that there is an association between the expression of AR in ceratohyoid and bicep muscles, which control dewlap display and pushup behaviors, respectively, and the frequency of the type of display they control. Additionally, I predicted that species with high pushup frequency and lower dewlap extension frequencies have a higher expression of AR in their bicep muscles, whereas species with higher dewlap extension frequencies and lower pushup frequencies have a higher AR expression in their ceratohyoid muscles.

In order to quantify AR expression in the different muscles, I performed AR immunocytochemistry, a method which uses antibodies to detect specific proteins. This allowed for visualization of the proportion of nuclei in muscle fibers from the ceratohyoid and bicep muscles which express high quantities of AR protein. Muscle fibers are formed by the fusion of multiple myoblasts during development, which makes them multinucleated. I quantified AR expression as the percentage of total nuclei that express AR protein to determine the association between AR expression and display behavior in anoles.

MATERIALS AND METHODS

Observational Data

I conducted behavioral observations on adult male lizards of each of three *Anolis* species: *A. coelestinus* (n = 20), *A. cybotes* (n = 30), and *A. brevirostris* (n= 41) during the summer breeding season in July 2011. These observations were performed in the lizards' natural habitat, on the grounds of Coralsol Resort in La Cienaga, Barahona, in southwestern Dominican Republic. Observations occurred between 0700 and 1800, and never during inclement weather (i.e., rain), as lizards may take refuge during those times (Hertz et al. 1993). I observed each individual for 30-60 min, for a total of 60 h of observational data (> 10 h/species). I recorded each instance of a display behavior (dewlap extensions and pushups), and measured the total duration of display time during the observational period. I also recorded locomotor behaviors (crawling, running, and jumping), foraging and copulation events. I calculated the average rate of dewlap and pushup displays from these observations for use in statistical analysis. In addition, I obtained data on rates of display behavior for three additional species (*A. olssoni*, *A. bahorucoensis*, and *A. carolinensis*) from Johnson (2007) and Johnson et al. (2010). These behavioral data were collected using the same methodology I used in this study.

Arena Trials

In natural behavioral observations, anole lizards may use dewlap and pushup displays in multiple contexts (courtship, territoriality, predator avoidance; see references in Introduction). To determine if ratios of dewlap:pushup displays in controlled male-male interactions were consistent with the lizards' natural displays, I conducted staged arena trials (Lailvaux et al., 2004; Perry et al., 2004). In these trials I paired two conspecific males that were caught by hand

or noose from the Coralsol Resort grounds and temporarily held in plastic bags until their trials began, after which they were released near their site of capture.

Before a trial began, I simultaneously placed two size-matched lizards under opaque containers on either side of a 12''x12''x11'' mesh butterfly cage containing a single wooden perch in the middle. Because these species are arboreal, they prefer to perch on a vertical substrate, rather than the horizontal surface at the bottom of the cage. Therefore, the presence of one perch promotes direct interaction (and thus display) between the males, as they compete for the perch. Before the trial began, the lizards were allowed to acclimate to the arena, with the opaque container blocking visual contact with the other lizard. After 5 min of acclimatization, I removed the containers and observed the lizards over a period of 10 min. To minimize observer effect on the lizards, the observers sat 3 m from the cages and remained motionless throughout the observation period. Consistent with the natural observational data, I recorded the number of dewlap extensions and pushups, and the total time spent displaying.

Tissue Acquisition

After collecting all behavioral data, I captured all *A. brevirostris*, *A. coelestinus*, *A. cybotes*, and *A. olssoni* to be used for tissue analysis by hand at night on July 11, 2011, on the grounds surrounding Coralsol Resort, the same areas where the natural behavioral observations took place; and *A. bahorucoensis* on July 11, 2011 in the mountainous region near Polo, Dominican Republic, in the same location of the behavioral observations reported in Johnson and Wade (2010). The individuals for which behavioral data were collected were not the same as those used for tissue analysis. Lizards were kept in air-filled plastic bags upon capture, and

moved to newspaper-filled cloth bags for transport (for thermal insulation and physical stability). Lizards were immediately dissected upon arrival at Trinity University (two days after capture).

In the laboratory, I measured snout-vent length (SVL) for each captured lizard using Mitutoyo digital calipers (± 0.005 cm). In addition, I measured each lizard's mass (to the nearest 0.1g) using Pesola spring scales. Lizards were then euthanized via rapid decapitation. Muscles from the jaw (ceratohyoid) and forelimb (biceps) were immediately harvested, in addition to kidneys to be used as a positive control for AR immunoreactivity. [In lizards, renal sex segments in the kidneys perform a similar role as the mammalian prostate gland, and enlarge in response to androgen (Winkler & Wade, 1998; Crews, 1980; Cueller et al., 1972). Due to the high androgen sensitivity in the kidneys for this function, there is a high expression of AR protein that we can use as a control for our antibody's immunoreactivity.] All tissues were then flash frozen on dry ice, and stored at -80°C.

Western Blot

I planned to assay AR expression in the biceps, ceratohyoid, and kidneys through their immunoreactivity to C-19 rabbit polyclonal antibody (Santa Cruz Biotechnology), which is known to bind to androgen receptor proteins across mammalian, avian, and reptilian taxa (Santa Cruz Biotechnology, J. Wade, pers. comm.). As a preliminary assessment of AR immunoreactivity across the anole species in this study, I performed a western blot using muscle and kidney tissue to confirm and quantify C-19 antibody reactivity with anole AR protein. All steps were carried out in room temperature unless otherwise specified. I used jaw and kidney tissues from all five Dominican Republic species, and arm tissues from three: *A. brevirostris*, *A. coelestinus* and *A. cybotes*. Tissues were thawed on ice for 30 min and ground on RIPA lysis

buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with a handheld tissue grinder. The samples were spun at 4 °C for 3 min at 7500 rpm. The protein was quantified from the supernatant by a bicinchoninic acid (BCA) assay on a Spectramax M4 (Molecular Dynamics).

To normalize total protein content in each sample, I prepared 18 µL samples with 20 µg of our protein lysate and 3 µL of Laemmli SDS Sample buffer in water and loaded the samples on a BioRad Mini-PROTEAN TGX precast gel. Gels were run for 2 h at 80 V, soaked in in transfer buffer for 15 min, and then assembled for transfer to PVDF membranes. The proteins were transferred to the membrane overnight at 25V at 4 °C. Membranes were washed 4 times with TBST for 5 min at room temperature, then blocked with 5% non-fat milk in TBST for 45 min on a shaker at 4 °C. Membranes were washed 3 times for 5 min in TBST and incubated in C-19 rabbit polyclonal antibody (2 µg) in 10 mL TBST overnight at 4 °C. Membranes were then washed 4 times in TBST for 10 min, and incubated with donkey anti-rabbit secondary antibody (5 µL antibody in 15 mL 5% non-fat milk in TBST) for 60 min. Membranes were washed 4 times in TBST for 5 min, then incubated with 10 mL WestPico chemiluminescent solution and imaged on ChemiDoc. I used ImageJ to calculate pixel density as a quantification of the amount of immunoreactivity in each tissue.

Tissue sections

Muscle and kidney tissues to be used for AR expression measurement via immunocytochemistry (ICC) and morphological measurements via Hematoxylin & Eosin (H&E) staining were sectioned with a Leica cryostat at 20 µm in 6 series (i.e., multiple sections were collected on a single slide at 120 µm intervals) and stored at -80 °C. The medial portion of each

tissue was sectioned on a transverse plane, such that cross-sections of the tissues were examined. ICC and H&E staining were carried out on alternate sections, with corresponding slides containing sections within 20 μm of each other.

Immunocytochemistry

Androgen receptor ICC was performed following Holmes and Wade (2005). Slides were air dried for 20 min, then fixed for 8 min in 4% paraformaldehyde in 0.1M phosphate buffered saline (1X PBS). Slides were rinsed 3 times for 5 min each in 1X PBS between every step. Slides were incubated for 30 min in 0.5% H_20_2 to remove endogenous peroxidase, then incubated for 1 h in 4% normal donkey serum in 1X PBS with 0.2% Triton X-100. Slides were incubated for 48 h in C-19 rabbit polyclonal antibody (2 μg/ml for throat and arm tissue) in 0.1M PBS with 0.2% Triton X-100 at 4 °C, and were then incubated in biotinylated donkey anti-rabbit secondary antibody (dilution 1 : 500 in 1X PBS) for 90 min. Slides were incubated for 1 h in Elite ABC solution (Vector Laboratories, Burlingame, CA, USA, Vectastain kit), then incubated for 7 min in nickel-enhanced diaminobenzidine (DAB) to visualize androgen receptors. Tissues were dehydrated (treated 1 min in 70% ethanol (EtOH), 5 min in dH_2O, 1 min in 70% EtOH, 1 min in 95% EtOH, 2x 1 min in 100% EtOH, 2x 5 min in xylene) and coverslipped using DPX.

In order to verify that AR+ nuclei staining was due to C-19 antibody, I ran an ICC control with muscle and kidney tissues. In this control, all steps of the ICC protocol were carried out in the same manner as described above, except that no primary antibody was added during the overnight incubation step. This allowed visualization of any background staining that might be occurring that was not caused by C-19 immunoreactivity to AR protein, and the extremely

low staining in this treatment confirmed that the staining of myonuclei in the complete ICC was primarily the result of AR first binding to the primary antibody.

Morphology stain

I performed H&E stains to determine total myonuclei counts in the ceratohyoid and bicep tissues, and to measure the fiber size of the muscles. Hematoxylin stains nucleic acids (and thus nuclei) purple, and eosin stains cytoplasm a lighter pink. Muscle cells are multinucleated, so this allowed for a total count of all myonuclei in the muscle tissues studied. Slides were air dried for 20 min, then dehydrated in 70% EtOH for 1 min and rehydrated in dH_2O for 1 min. Slides were then stained with Harris hematoxylin for5 min. Slides were dipped in dH_2O for a few seconds, then stained with 30% eosin in 70% EtOH for 30 sec. Slides were then dehydrated (treated a few seconds successively in 70% EtOH, 95% EtOH, twice in 100% EtOH, and 2x 5 min in xylene), then coverslipped using DPX.

Tissue analysis

To determine the proportion of AR immunoreactive (AR+) nuclei, I counted AR+ nuclei from ICC stained sections and the total number of all nuclei from H & E stained sections for each lizard (Figure 2). Photographs of the ICC and H& E stained sections were taken with a Hitachi HV-C20 3CCD camera on a Leica Axioskop 2 microscope at 100X magnification for both the ceratohyoid and bicep muscles. These photographs were used to manually count the average number of AR+ nuclei per cell on both ICC- and H&E-stained slides. The proportion of AR+ nuclei was then calculated by dividing the number of AR+ nuclei by the total number of myonuclei.

In addition to nuclei counts, the muscle fiber size mean for biceps and triceps was also calculated. The same pictures used for H&E myonuclei counts were used in ImageJ to obtain the cross-sectional area of 20 arbitrarily chosen muscle fibers in the medial portion of the ceratohyoid and bicep muscles. These measures were then averaged to obtain the mean muscle fiber size for each of the muscles for each individual.

Statistical analysis

To determine if the species differed in display rates, ratio of pushup:dewlap per display, proportion of AR+ nuclei in each muscle, and fiber size in each muscle, I used a series of Analyses of Variance (ANOVA) followed by Tukey's HSD post hoc tests. To determine the interspecific relationships between behavior measures and muscle traits, I used multiple linear regression analyses. In the first, dewlap extension rate was the dependent variable, and proportion of AR+ nuclei in the ceratohyoid and ceratohyoid muscle fiber size were the independent variables. Another regression analysis considered pushup frequency as the dependent variable, and proportion of AR+ nuclei in the biceps and bicep muscle fiber size as the independent variables.

Because standard statistical analyses consider all data to be independent, and the shared evolutionary history of species violate this assumption, I also used phylogenetically controlled analyses to determine whether AR expression in the ceratohyoid and dewlap display rates evolved in association with each another. Using the independent contrast method of Felsenstein (1985), and the Rabosky and Glor (2010) phylogeny of anoles, trimmed to include only the species being studied, contrasts for %AR+ nuclei and display rates were calculated in R using the program APE (Paradis et al. 2004). The contrasts were used in regression analyses to determine

the relationship between AR expression and dewlap display rate. I did not do a comparable analysis using bicep measures and pushup rates because data from only five species were available, and as independent contrasts reduce the degrees of freedom in an analysis by 1 (Felsenstein 1985), this analysis would require a regression using only four data points.

RESULTS

Behavioral observations

My observations of natural behavior in three species, combined with previously reported data on three others (see Materials and Methods) confirmed that the species differed in the proportions of dewlap extensions to pushups in their displays. The three focal species differed in dewlap display rates, with *A. coelestinus* having the lowest display rate, followed by *A. cybotes*, and *A. brevirostris* having the highest dewlap display rate ($F_{2,90} = 12.904$, $p < 0.001$). These three species also differed in pushup display rates, with *A. brevirostris* performing fewer pushups than *A. coelestinus* and *A. cybotes* ($F_{2,90} = 4.547$, $p = 0.013$). In addition, *A. coelestinus* and *A. cybotes* performed far more pushups than dewlap extensions in their displays compared to *A. brevirostris* ($F_{2,83} = 10.2$, $p < 0.001$). *Anolis bahorucoensis*, *A. olssoni*, and *A. carolinensis* were not observed in this study, but averages of these species were obtained from previous studies and are included in Figure 3a for comparison with my observational data. In addition, I found that behavior in arena trials was generally consistent with natural display behavior, showing that natural displays are representative of aggressive displays performed during male-male conflict, although *A. coelestinus* displayed very rarely in the arena trials (Figure 3b).

Western blot analysis

The pilot study of AR immunoreactivity across the species of anoles studied here suggested that the C-19 antibody was strongly reactive in each species (results not shown). A Western blot analysis that includes actin normalization will further determine whether these species are equally immunoreactive with C-19 antibody.

Species differences in muscle physiology

The percentage of AR+ nuclei in the ceratohyoid did not differ among the five species for which morphology was measured (i.e., excluding *A. carolinensis* $F_{4,36} = 0.737$, $p = 0.573$, Table 1). However, there was a significant difference among species in the AR+ nuclei content of the bicep muscles ($F_{4,36} = 3.081$, $p = 0.028$- Table 1), with Tukey's HSD post hoc tests showing that *A. brevirostris* exhibited more AR+ nuclei than *A. coelestinus*, and the other three species not differing from any of the five.

The five species differed in muscle fiber size (ceratohyoid: $F_{4,36} = 29.2$, $p < 0.001$; bicep: $F_{4,36} = 10.575$, $p < 0.001$; Table 1). Tukey's post hoc tests showed that *A. bahorucoensis* had the smallest ceratohyoid muscle fibers, followed by *A. coelestinus* and *A. brevirostris*. *Anolis cybotes* and *A. olssoni* had the largest ceratohyoid muscle fibers. For the bicep muscle, Tukey's post hoc tests showed that *A. olssoni* and *A. bahorucoensis* had smaller bicep muscle fibers than *A. brevirostris, A. cybotes* and *A. coelestinus*. Species also differed in the two measures of body size: mass ($F_{4,36} = 79.75$, $p = 0.028$) and snout-vent length ($F_{4,36} = 177.645$, $p < 0.001$; Table 1). *Anolis bahorucoensis*, *A. olssoni* and *A. brevirostris* were smaller than *A. coelestinus* and *A. cybotes* for both of these measures.

Interspecific relationships between behavior and muscle physiology

The percentage of AR+ cells in ceratohyoid and bicep muscles were significantly correlated ($r = 0.990$; $p < 0.001$; Table 2, Figure 4), such that species with higher percentages of AR+ nuclei in one muscle also have more AR+ nuclei in the other muscle. When I used only the data I collected on the five Dominican Republic species, there was no relationship between proportion of AR+ nuclei in the ceratohyoid muscle and dewlap extension rates ($r = 0.577$, $p =$

0.308; Figure 5). However, when previously-collected AR data on *A. carolinensis* (Holmes & Wade, 2005) were included with our analyses, there was a significant positive correlation between proportion of AR+ nuclei in the ceratohyoid muscle and dewlap extension rates ($r = 0.965$, $p = 0.002$; Figure 6). An analysis using phylogenetic independent contrasts for %AR and dewlap display frequencies for the five focal species and *A. carolinensis* showed that there was a significant relationship between these two variables ($R^2 = 0.914$, $F_{4,1} = 53.87$, $p = 0.0018$; Figure 7), indicating that the evolution of increased display rate was associated with the evolution of increased AR+ nuclei in the ceratohyoid.

There was no significant correlation between the percentage of AR+ nuclei in bicep muscles and pushup rates ($r = -0.585$, $p = 0.301$; Figure 8).

While ceratohyoid muscle fiber size was not related to dewlap extension frequency ($r = 0.149$, $p = 0.778$, Figure 9), bicep muscle fiber size was significantly related to pushup frequency ($r = 0.878$, $p = 0.050$) (Figure 10). Additionally, bicep fiber size was also significantly related to mass ($r = 0.901$, $p = 0.037$, Figure 11), with bigger lizards having larger areas in their bicep muscle fibers.

DISCUSSION

Studies of animal behaviors and the mechanisms underlying them that have focused on individual species have informed our understanding of the way structures and functions are associated in immensely valuable ways. However, to truly understand the relationships between behavioral and physiological traits in broader contexts, it is important to carry out studies of behavioral mechanisms in groups of closely related species. For this purpose, the *Anolis* lizards I investigated are a very suitable focal group. Due to the similarities across species in morphology, ecology, and social communication behavior (Losos 2009), it was possible to directly compare the morphological characters of multiple species and their associated behaviors.

The average display rates found during behavioral observations were generally consistent with those found previously in these species (Johnson & Wade, 2010). This suggests that the patterns of social display behavior are consistent across time and space, as my field study was performed in a different year and at different localities than those previously studied. In addition, the average ratio of dewlap extensions to pushups was similar in natural observations and arena trials, suggesting that the preferred method of display remains consistent in different contexts.

Because male dewlaps are used in a variety of different social contexts, seeing differences in dewlap display rates can be due to multiple reasons. These displays can be used for courtship, territoriality and predator deterrence, and it is likely that these factors vary significantly in the way they affect each species. Because the dewlap is used occasionally in predator deterrence, a more highly-predated species may exhibit increased display rates. Alternatively, high rates of dewlap display could make an individual more vulnerable to predation, if use of the dewlap in social display alerts a predator to the location of the individual.

Further, a species with higher density, and thus higher rates of encounters with conspecifics, may lead to increased display rates for its individuals. While no relationship has been found between higher population densities and dewlap display frequencies across species, there was a relationship between the visibility of a species' habitat and the frequency of its display behavior (Johnson et al. 2010), and so the visual environment of each species may play an important role in the frequency of its display. As these different factors lead species to evolve particular display behaviors, the underlying muscle structures controlling them may evolve in association with the behaviors in their capacity to support these display patterns.

I expected to find that AR expression in muscles controlling each display behavior was associated with the frequency of use of that specific display. Using a standard (i.e, non-phylogenetic) statistical analysis, we found no such relationship among our five focal species. However, there was a significant relationship between these characters when considered under phylogenetic constraints including all six species for which data were available (Figure 7). This suggests that AR expression and display behavior have evolved in association across the different anole lineages studied. It is important to note that while this does not directly implicate a causal relationship between the two factors, there is no relationship between muscle fiber size, fiber type composition, circulating androgen levels or seasonality and display behavior. Considering these factors together, our data do suggest a causal relationship between AR and display behavior.

The different effects that AR can have on a muscle cell upon binding to testosterone are not fully known. It is known that AR's main function is to bind to DNA and act as transcription factors. While many developmental genes have been associated with AR regulation, little is currently known about the immediate activational effects that AR can have on adults. One of the

known effects associated with increased AR expression is higher regeneration rates in that muscle (Serra et al. 2013). It is one possibility that as display behavior evolved to be more frequent in different species, the expression of AR in the display-controlling muscle also increased in order to keep that muscle healthy and support its constant use.

Consistent with previous work, ceratohyoid fiber size was not associated with dewlap extension frequency across anole species (Johnson et al. 2010). Interestingly, bicep fiber size, which had not previously been studied in these species, was positively associated with pushup frequency (Figure 10). There is a general trend across animal taxa where structures used more frequently tend to evolve to become larger (reviewed in Johnson & Wade 2010). It is interesting that this trend holds for bicep muscles and pushup behavior, but not ceratohyoid muscles for dewlap extensions.

In addition to pushup frequency, bicep muscle fiber size was also related to the lizard's total mass (Figure 11). These two trends suggest an important difference between the bicep and ceratohyoid muscle. The ceratohyoid muscle's main purpose is the extension of the ceratobranchial cartilage, which remains largely unaffected by the individual's overall mass. The bicep muscles must work to move the entirety of the lizard's body, not just a cartilage. This suggests that more frequent pushup behavior or larger lizards require more robust muscles to carry out this function.

AR expression and muscle fiber size inform our understanding of the link between morphology and behavior both within and across species. However, given the complexity of AR interactions in the cell it is important to study not only their expression and patterns of distribution, but also the specific mechanism of action and their immediate effects upon

interaction with testosterone. The general relationship between AR and the behaviors it mediates should be further elucidated by studies looking at AR expression patterns in other regions (such as specific brain tissues) and a more robust understanding of the specific cellular interactions occurring upon AR activation.

REFERENCES

Adkins E, Boop J, Koutnik D, Morris J, Pniewski E. Further evidence that androgen aromatization is essential for the activation of copulation in male quail. Physiol Behav 1980;24(3):441-6.

Adkins E, Schlesinger L. Androgens and the social behavior of male and female lizards (*Anolis carolinensis*). Horm Behav 1979;13(2):139-52.

Adkins-Regan E. Hormones and animal social behavior. Monographs in Behavior and Ecology 2005.

Arnold AP. The effects of castration and androgen replacement on song, courtship, and aggression in zebra finches (*Poephila guttata*). J Exp Zool 1975;191(3):309-25.

Arnold AP, Breedlove SM. Organizational and activational effects of sex steroids on brain and behavior: A reanalysis. Horm Behav 1985;19(4):469-98.

Balthazart J, Foidart A, Wilson EM, Ball GF. Immunocytochemical localization of androgen receptors in the male songbird and quail brain. J Comp Neurol 1992;317(4):407-20.

Brantley RK, Wingfield JC, Bass AH. Sex steroid levels in *Porichthys notatus*, a fish with alternative reproductive tactics, and a review of the hormonal bases for male dimorphism among teleost fishes. Horm Behav 1993;27(3):332-47.

Breedlove SM, Jordan CL, Kelley DB. What neuromuscular systems tell us about hormones and behavior. Hormones, Brain and Behavior, Academic Press, New York 2002;4:193-22.

Crews D. Interrelationships among ecological, behavioral, and neuroendocrine processes in the reproductive cycle of *Anolis carolinensis* and other reptiles. Advances in the Study of Behavior 1980;2:1-74.

Crews D. Endocrine control of reptilian reproductive behavior. Endocrine Control of Reptile Reproductive Behavior (Ed.by C.Beyer), Raven Press, New York 1979:167-222.

Crews D, Moore MC. Evolution of mechanisms controlling mating behavior. Science 1986;231(4734):121-5.

Cuellar HS, Roth JJ, Fawcett JD, Jones RE. Evidence for sperm sustenance by secretions of the renal sexual segment of male lizards, *Anolis carolinensis*. Herpetologica 1972:53-7.

Dunlap K, Thomas P, Zakon H. Diversity of sexual dimorphism in electrocommunication signals and its androgen regulation in a genus of electric fish, *Apteronotus*. Journal of Comparative Physiology A 1998;183(1):77-86.

Dunlap KD, Zakon HH. Behavioral actions of androgens and androgen receptor expression in the electrocommunication system of an electric fish, *Eigenmannia virescens*. Horm Behav 1998;34(1):30-8.

Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 1985:783-91.

Greenberg B, Noble GK. Social behavior of the American chameleon (*Anolis carolinensis*). Physiol Zool 1944;17(4):392-439.

Harding CF, Sheridan K, Walters MJ. Hormonal specificity and activation of sexual behavior in male zebra finches. Horm Behav 1983;17(1):111-33.

Hart B. L.(1967). Testosterone regulation of sexual reflexes in spinal male rats. Science;155:1283-4.

Herbst KL, Bhasin S. Testosterone action on skeletal muscle. Current Opinion in Clinical Nutrition & Metabolic Care 2004;7(3):271-7.

Holmes M, Wade J. Testosterone regulates androgen receptor immunoreactivity in the copulatory, but not courtship, neuromuscular system in adult male green anoles. J Neuroendocrinol 2005;17(9):560-9.

Huddleston GG, Song CK, Paisley JC, Bartness TJ, Clancy AN. Gonadal steroid receptors colocalize with central nervous system neurons projecting to the rat prostate gland. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 2007;292(6):R2196-205.

Jenssen TA. Evolution of anoline lizard display behavior. Am Zool 1977;17(1):203-15.

Johnson MA. Behavioral ecology of Caribbean *Anolis* lizards. Washington University, St. Louis, Missouri 2007.

Johnson MA, Wade J. Behavioural display systems across nine *Anolis* lizard species: Sexual dimorphisms in structure and function. Proceedings of the Royal Society B: Biological Sciences 2010;277(1688):1711-9.

Kodric-Brown A, Brown JH. Truth in advertising: The kinds of traits favored by sexual selection. Am Nat 1984:309-23.

Lailvaux SP, Herrel A, VanHooydonck B, Meyers JJ, Irschick DJ. Performance capacity, fighting tactics and the evolution of life–stage male morphs in the green anole lizard (*Anolis carolinensis*). Proceedings of the Royal Society of London.Series B: Biological Sciences 2004;271(1556):2501-8.

Leal M, Rodriguez-Robles JA. Signalling displays during predator–prey interactions in a Puerto Rican anole, *Anolis cristatellus*. Anim Behav 1997;54(5):1147-54.

Losos JB. Lizards in an evolutionary tree: Ecology and adaptive radiation of anoles. Univ of California Press; 2009.

Lovern MB, Holmes MM, Fuller CO, Wade J. Effects of testosterone on the development of neuromuscular systems and their target tissues involved in courtship and copulation in green anoles (*Anolis carolinensis*). Horm Behav 2004;45(5):295-305.

Lovern MB, Holmes MM, Wade J. The green anole (*Anolis carolinensis*): A reptilian model for laboratory studies of reproductive morphology and behavior. Ilar Journal 2004;45(1):54-64.

Lu NZ, Wardell SE, Burnstein KL, Defranco D, Fuller PJ, Giguere V, Hochberg RB, McKay L, Renoir J, Weigel NL. International union of pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: Glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev 2006;58(4):782-97.

Mason P, Adkins EK. Hormones and social behavior in the lizard, *Anolis carolinensis*. Horm Behav 1976;7(1):75-86.

McGinnis MY, Dreifuss RM. Evidence for a role of testosterone-androgen receptor interactions in mediating masculine sexual behavior in male rats. Endocrinology 1989;124(2):618-26.

Meisel R, Sachs B. The physiology of male sexual behavior. The Physiology of Reproduction 1994;2:3-105.

Miles D, Sinervo B, Hazard L, Svensson E, Costa D. Relating endocrinology, physiology and behaviour using species with alternative mating strategies. Funct Ecol 2007;21(4):653-65.

Mooradian AD, Morley JE, Korenman SG. Biological actions of androgens. Endocr Rev 1987;8(1):1-28.

Neal JK, Wade J. Courtship and copulation in the adult male green anole: Effects of season, hormone and female contact on reproductive behavior and morphology. Behav Brain Res 2007;177(2):177-85.

Nicholson KE, Harmon LJ, Losos JB. Evolution of *Anolis* lizard dewlap diversity. PLoS One 2007;2(3):e274.

O'Bryant EL, Wade J. Sexual dimorphisms in a neuromuscular system regulating courtship in the green anole lizard: Effects of season and androgen treatment. J Neurobiol 1999;40(2):202-13.

Paradis E., Claude J. & Strimmer K. APE: Analyses of phylogenetics and evolution in R language. Bioinformatics 2004;20:289-290.

Perry G, LeVering K, Girard I, Garland Jr T. Locomotor performance and social dominance in male *Anolis cristatellus*. Anim Behav 2004;67(1):37-47.

Rabosky DL, Glor RE. Equilibrium speciation dynamics in a model adaptive radiation of island lizards. Proceedings of the National Academy of Sciences 2010;107(51):22178-83.

Saartok T, Dahlberg E, Gustafsson J. Relative binding affinity of anabolic-androgenic steroids: Comparison of the binding to the androgen receptors in skeletal muscle and in prostate, as well as to sex hormone-binding globulin. Endocrinology 1984;114(6):2100-6.

Serra C, Tangherlini F, Rudy S, Lee D, Toraldo G, Sandor NL, Zhang A, Jasuja R, Bhasin S. Testosterone improves the regeneration of old and young mouse skeletal muscle. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 2013;68(1):17-26.

Shelley DN, Choleris E, Kavaliers M, Pfaff DW. Mechanisms underlying sexual and affiliative behaviors of mice: Relation to generalized CNS arousal. Social Cognitive and Affective Neuroscience 2006;1(3):260-70.

Sinervo B, Miles DB, Frankino WA, Klukowski M, DeNardo DF. Testosterone, endurance, and darwinian fitness: Natural and sexual selection on the physiological bases of alternative male behaviors in side-blotched lizards. Horm Behav 2000;38(4):222-33.

Winkler SM, Wade J. Aromatase activity and regulation of sexual behaviors in the green anole lizard. Physiol Behav 1998;64(5):723-31.

TABLES

Table 1: Sample size (n = 49) average body size (mass and SVL), muscle fiber and muscle %AR+ nuclei measures for the *Anolis* lizard species in this study. *Anolis carolinensis* data from Holmes & Wade (2005) and Johnson & Wade (2010).

Species	N	Mass (g)	SVL (mm)	CH fiber size (μm^2)	Bicep fiber size (μm^2)	CH %AR+ nuclei	Bicep %AR+ nuclei
A. bahorucoensis	7	1.09	42.29	592.97	1932.40	0.4164	0.3176
A. brevirostris	10	2.29	47.00	865.28	3386.07	0.4310	0.3552
A. coelestinus	10	6.10	65.30	841.41	4381.34	0.3690	0.2244
A. cybotes	10	7.00	60.70	1172.17	3957.40	0.3903	0.2524
A. olssoni	4	1.06	45.25	1200.20	1920.51	0.4324	0.3689
A. carolinensis	8	5.57	63.40	961.00		0.7857	

Table 2: Pearson correlations among morphological, physiological, and behavioral traits among the five focal species of *Anolis* lizards. Bold font indicates a significant correlation, * indicates $p < 0.05$, and ** indicates $p < 0.01$.

	SVL	%AR (CH)	%AR (Bicep)	Ceratohyoid Fiber Size (μm^2)	Bicep Fiber Size (μm^2)	Dewlap Rate	Pushup Rate
Mass	**0.952****	0.181	**-0.891***	0.295	**0.901***	0.219	0.664
SVL		0.316	-0.902*	0.254	**0.908***	0.354	0.803
%AR (CH)			**0.990****	0.055	-0.774	**0.965****	-0.649
%AR (Bicep)				0.065	-0.775	0.601	-0.585
Ceratohyoid Fiber Size					0.13	0.149	0.104
Bicep Fiber Size						0.036	0.878
Dewlap Rate							0.207

FIGURE LEGENDS

Figure 1: Phylogenetic relationship between the species examined, from Rabosky and Glor (2010), pruned to include only the species used in our study.

Figure 2: Representative AR ICC and H&E stains of the same ceratohyoid muscle fiber showing immunoreactive myonuclei and total myonuclei, respectively, in 20 μm sections from consecutive slides. Sections were taken on a cross-sectional plane from *A. carolinensis* jaw tissues. Myonuclei in H&E sections stained dark purple, and in ICC stained dark brown. A comparison of both images allows us to count the total number of myonuclei and AR+ nuclei from the same tissue.

Figure 3: a) Natural dewlap extension and pushup rates for the six species studied and b) dewlap extension and pushup rates for the three species for which arena trials were performed. Data for *A.brevirostris A. coelestinus* and *A. cybotes* are from this study; data for *A. bahorucoensis* and *A. olssoni* are from Johnson (2007); display data for *A. carolinensis* from Johnson & Wade (2010). Species names are abbreviated as follows: Ba, *A. bahorucoensis*; Br, *A. brevirostris*; Coe, *A. coelestinus*; Cy, *A. cybotes*; Ol, *A. olssoni*; Car, *A. carolinensis*.

Figure 4: The percentage of AR+ nuclei in ceratohyoid muscles was associated with the percentage of AR+ nuclei in bicep muscles for the five species studied ($r = 0.99$, $p < 0.001$).

Figure 5: Ceratohyoid %AR+ expression shows no relationship with dewlap display rate across species when only our five focal species are considered ($r = 0.577$, $p = 0.308$).

Figure 6: Ceratohyoid %AR+ expression increases with higher dewlap display rate across species, using all six species for which %AR and display data currently available (the five focal species and *A. carolinensis*; $r = 0.965$, $p = 0.002$).

Figure 7: Independent contrasts for ceratohyoid %AR+ expression increase in association with independent contrasts in dewlap display rate across species in a phylogenetically-controlled analysis, using all six species for which %AR and display data currently available (the five focal species and *A. carolinensis;* $R^2 = 0.914$, $F_{4,1} = 53.87$, $p = 0.0018$).

Figure 8: Bicep %AR+ expression shows no relationship with pushup display rate across the five species studied ($r = -0.585$, $p = 0.301$).

Figure 9: Ceratohyoid fiber size shows no association with dewlap display frequencies for the five species studied ($r = 0.149$, $p = 0.778$).

Figure 10: Bicep fiber size increases with pushup display frequencies for the five species studied ($r = 0.878$, $p = 0.050$).

Figure 11: Bicep fiber size increases with lizard mass for the five species studied ($r = 0.901$, $p = 0.037$).

FIGURES

Figure 1:

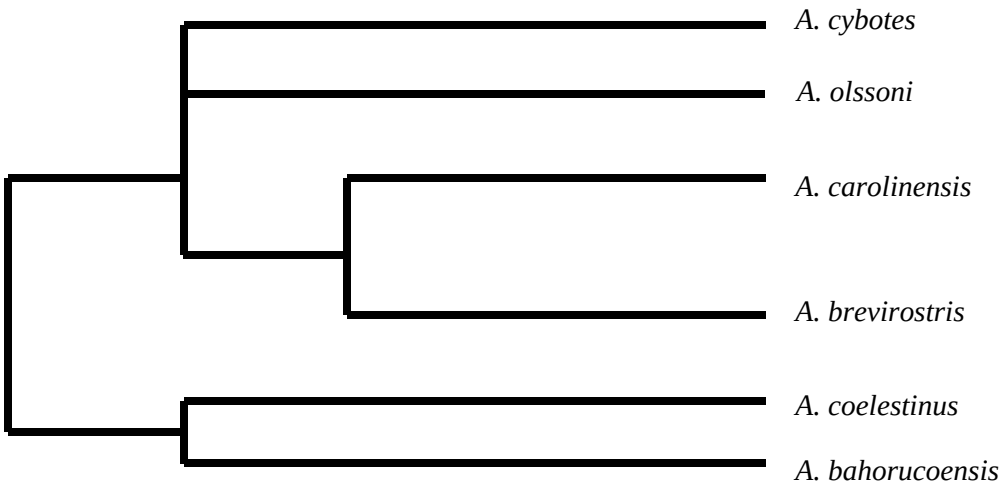
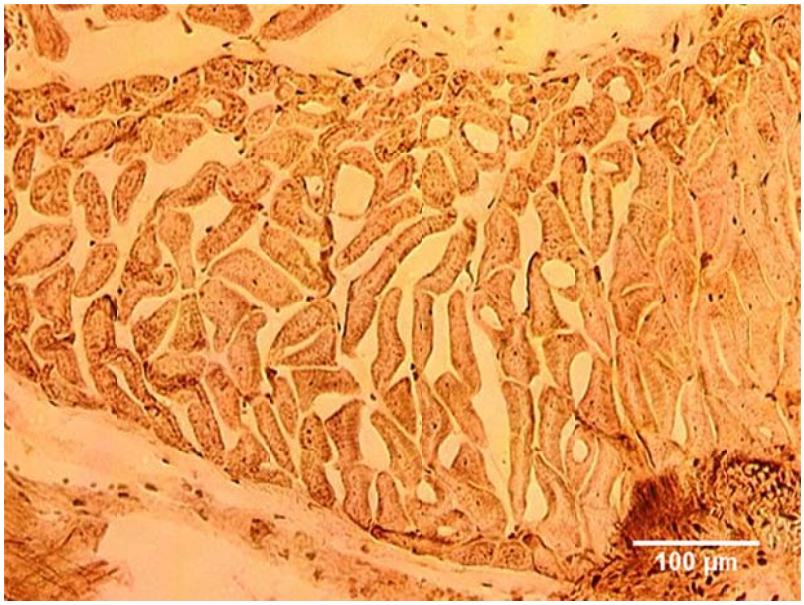


Figure 2:

ICC



H&E

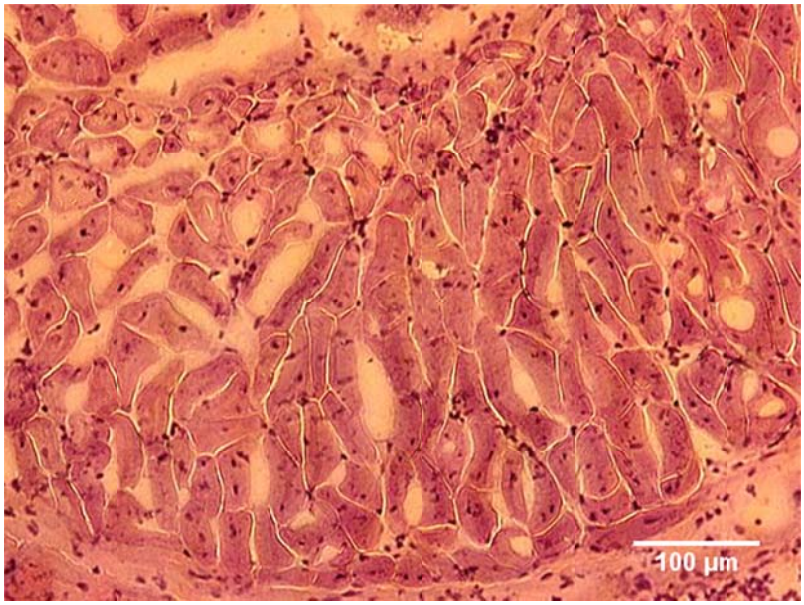


Figure 3a:

Natural Observations

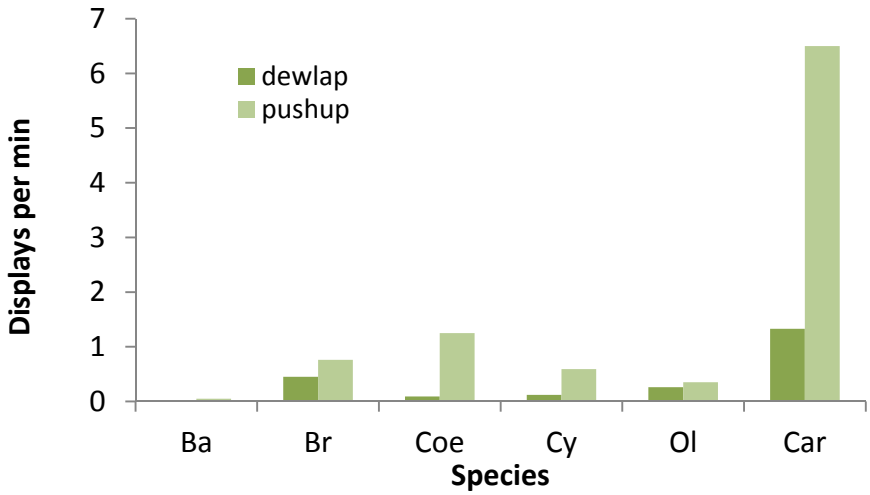


Figure 3b:

Arena Trials

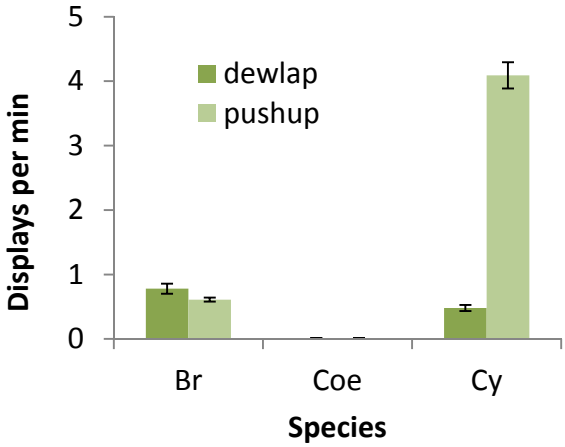


Figure 4:

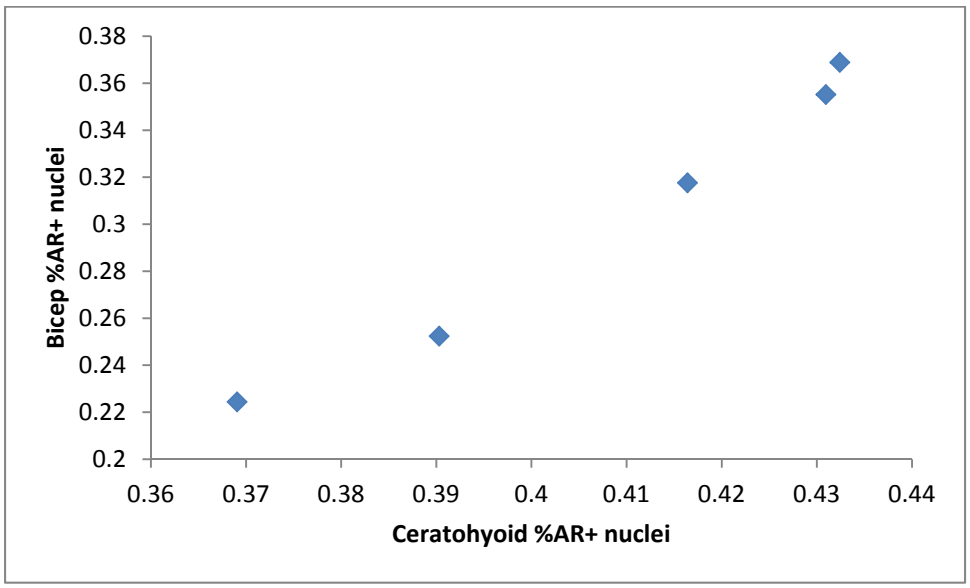

0.38
0.36
0.34
0.32
0.3
0.28
0.26
0.24
0.22
0.2
Bicep %AR+ nuclei
0.36
0.37
0.38
0.39
0.4
0.41
0.42
0.43
0.44
Ceratohyoid %AR+ nuclei

Figure 5:

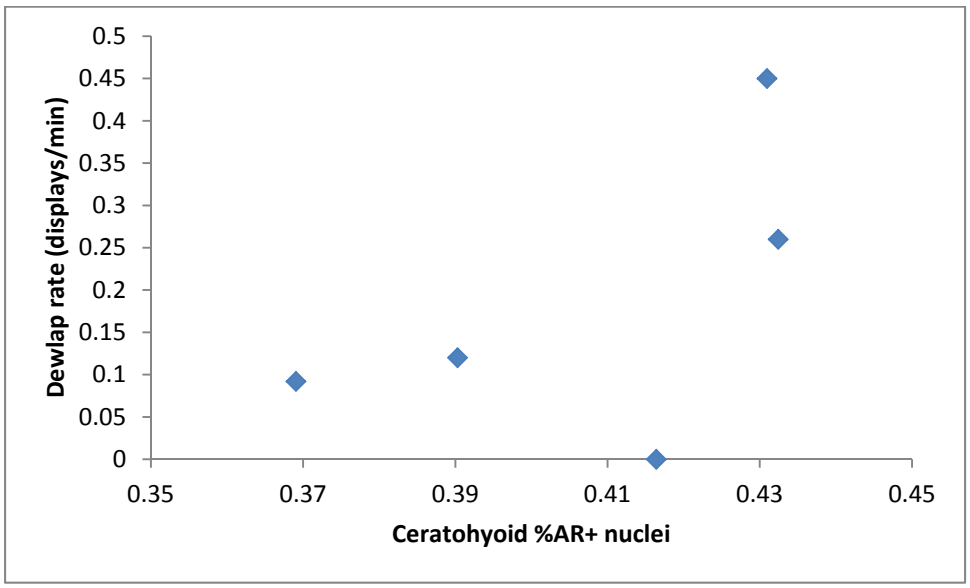

Dewlap rate (displays/min)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.35
0.37
0.39
0.41
0.43
0.45
Ceratohyoid %AR+ nuclei

Figure 6:

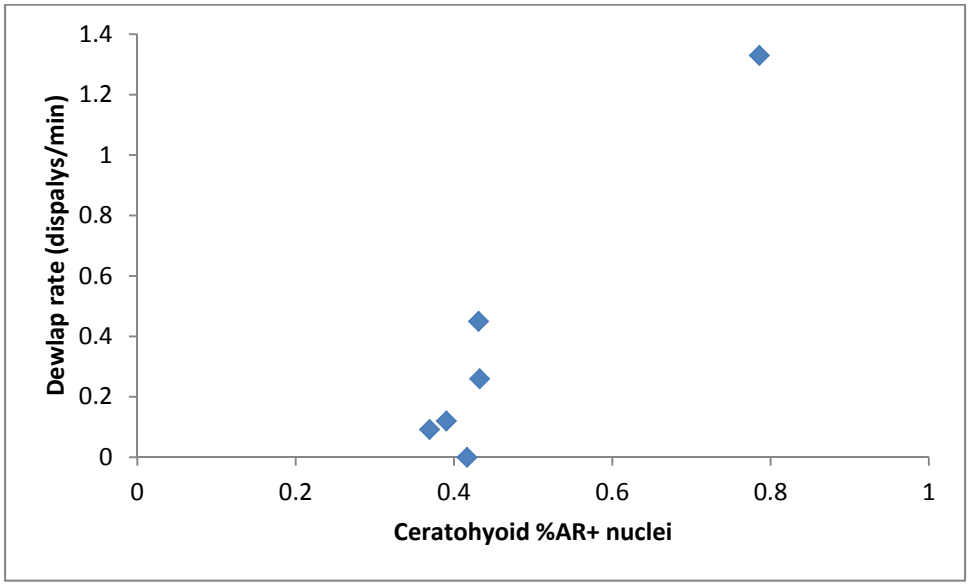

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Dewlap rate (dispalys/min)
0
0.2
0.4
0.6
0.8
1
Ceratohyoid %AR+ nuclei

Figure 7:

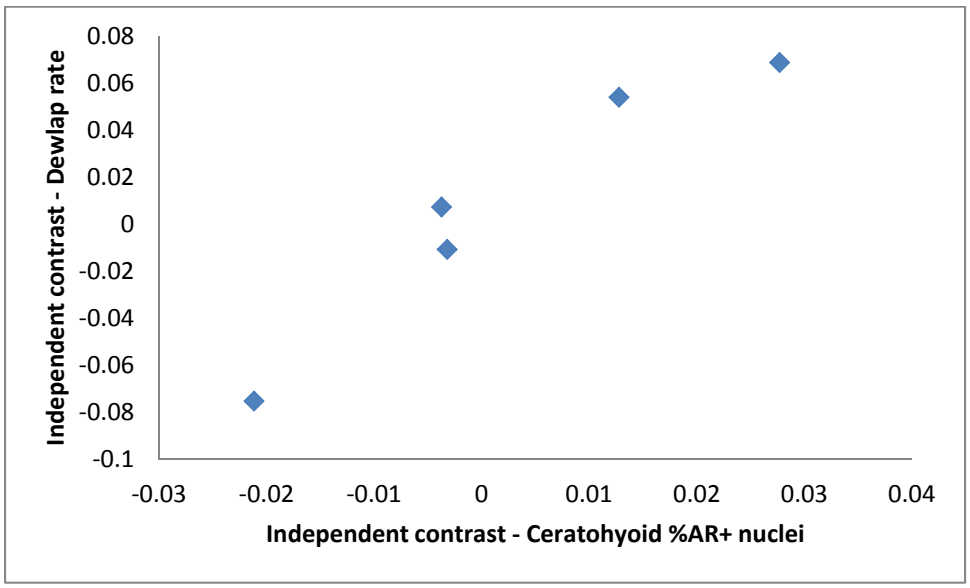

0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
-0.1
Independent contrast - Dewlap rate
-0.03
-0.02
-0.01
0
0.01
0.02
0.03
0.04
Independent contrast - Ceratohyoid %AR+ nuclei

Figure 8:

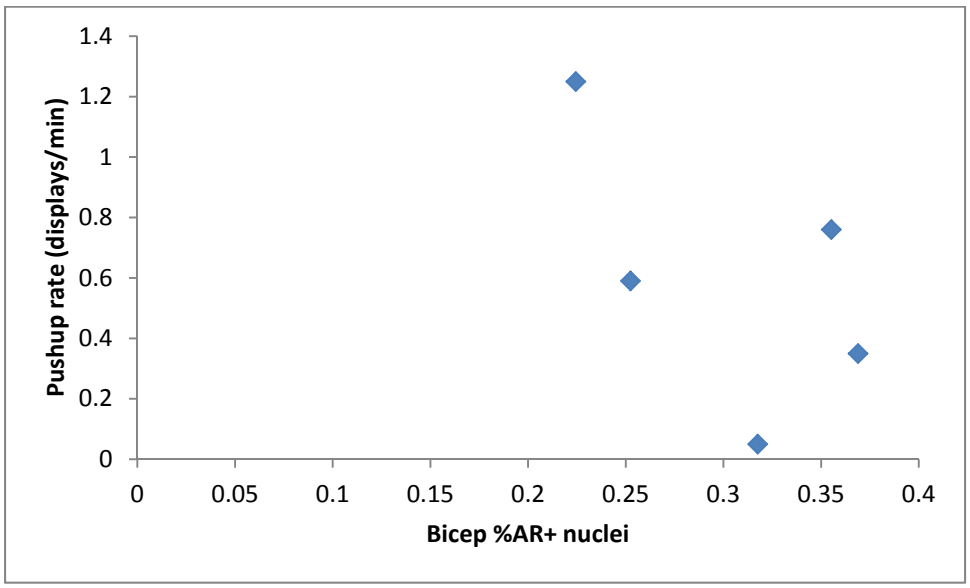

Pushup rate (displays/min)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
Bicep %AR+ nuclei

Figure 9:

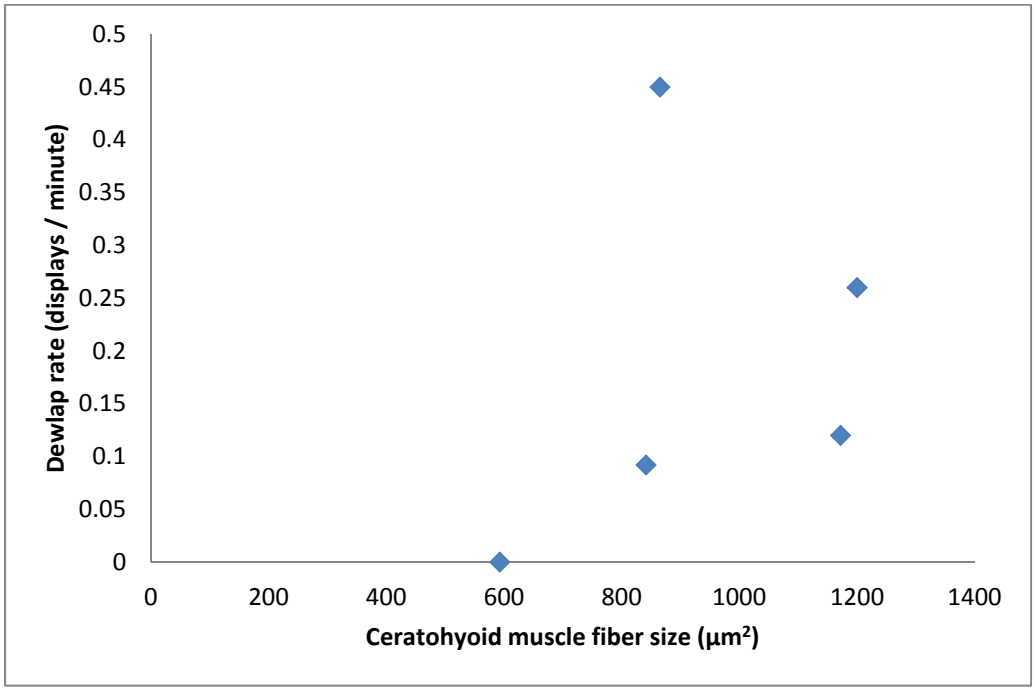

0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Dewlap rate (displays / minute)
0
200
400
600
800
1000
1200
1400
Ceratohyoid muscle fiber size (µm²)

Figure 10:

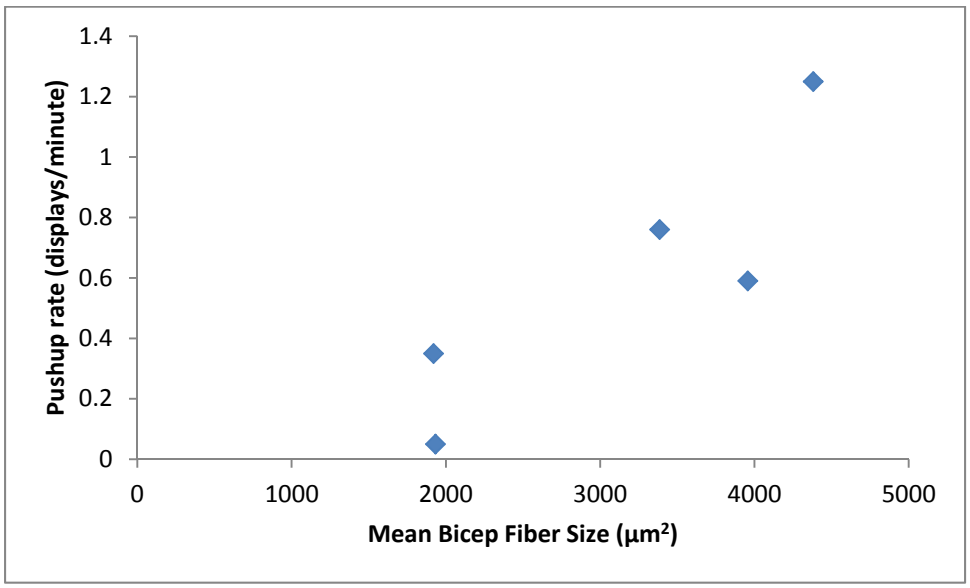
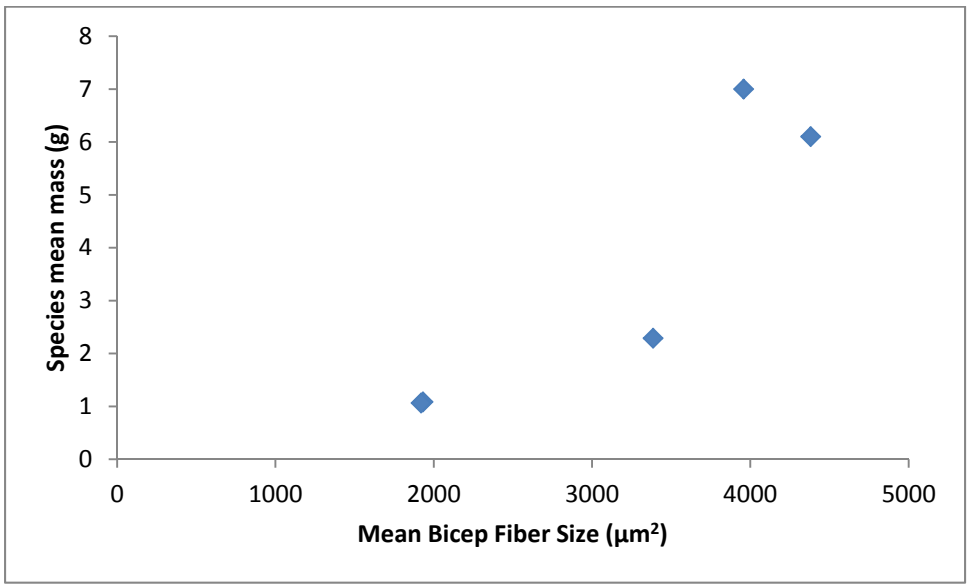

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Pushup rate (displays/minute)
0
1000
2000
3000
4000
5000
Mean Bicep Fiber Size (µm²)

Figure 11:


Species mean mass (g)
8
7
6
5
4
3
2
1
0
0
1000
2000
3000
4000
5000
Mean Bicep Fiber Size (µm²)