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Androgen receptor expression and morphology of forebrain and neuromuscular systems in male green anoles displaying individual differences in sexual behavior

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Abstract

Investigating individual differences in sexual performance in unmanipulated males is important for understanding natural relationships between behavior and morphology, and the mechanisms regulating them. Among male green anole lizards, some court and copulate frequently (studs) and others do not (duds). To evaluate potential factors underlying differences in the level of these behaviors, morphology and androgen receptor expression in neuromuscular courtship and copulatory structures, as well as in the preoptic area and amygdala, were compared in males displaying varying degrees of sexual function. This study revealed that individual differences in behavior among unmanipulated males, in particular the extension of a throat fan (dewlap) used during courtship, were positively correlated with the size of fibers in the associated muscle and with soma size in the amygdala. The physiological response to testosterone, as indicated by the height of cells in an androgen-sensitive portion of the kidney, was also correlated with male sexual behavior, and predicted it better than plasma androgen levels. Androgen receptor expression was not related to the display of courtship or copulation in any of the tissues examined. The present data indicate that higher levels of male courtship behavior result in (or are the result of) enhanced courtship muscle and amygdala morphology, and that androgen-sensitive tissue in studs may be more responsive to testosterone than duds. However, some mechanism(s) other than androgen receptor expression likely confer this difference in responsiveness.

Keywords

Testosterone; Courtship; Copulation; Reptile; Preoptic area; Amygdala

Introduction

Individual differences in male sexual behavior exist in a variety of species (e.g., Crews, 1998; Perkins et al., 1992). For example, in populations of rams, rats, guinea pigs, whiptail lizards and songbirds, some males court and copulate frequently (studs) and others do not (duds, Alexander et al., 1999; Bernard et al., 1996; Crews, 1998; Harding and Feder, 1976; Lindzey and Crews, 1992; Perkins et al., 1992; Portillo et al., 2007). These types of differences among unmanipulated males can be quite useful for understanding natural relationships between behavior and morphology. However, relatively few studies have identified specific mechanisms (e.g., Bernard et al., 1996; Lindzey and Crews, 1992; Perkins et al., 1992).

Previous investigations have focused on various physiological and morphological characteristics of forebrain regions and neuromuscular structures. Factors including neuron soma size, brain region volume, aromatase activity and estrogen receptor immunoreactivity are increased in the preoptic (POA) and amygdala (AMY) of rats and/or rams exhibiting high compared to low sexual activity (Alexander et al., 1993; Alexander et al., 2001b; Clark et al., 1985; Portillo et al., 2007; Prince et al., 1998). In addition, courtship neuromuscular structures are larger in groups of male sonic fish that vocalize than those that do not (Bass and Baker, 1990; Bass and Marchaterre, 1989; Bass and Zakon, 2005; Brantley et al., 1993), but motoneurons and muscle fibers important for copulation can be smaller in groups of copulating compared to non-copulating male rats (Breedlove, 1997; Raouf et al., 2000). In contrast, individual differences in whiptail lizard behavior do not appear related to POA or AMY volume (Wade and Crews, 1991; Wade et al., 1993). Similarly, plasma testosterone (T) levels do not explain individual differences in behavior in guinea pigs, rams or whiptail lizards (Alexander et al., 1999; Harding and Feder, 1976; Lindzey and Crews, 1992; Perkins et al., 1992).

The green anole lizard provides an excellent model for these types of investigations. A substantial range in reproductive performance is present in our colony, as approximately 5–10% of the male anoles rarely court or copulate with females. Male anoles exhibit characteristic reproductive displays, which are facilitated by T (Adkins and Schlesinger, 1979; Crews et al., 1978; Lovern et al., 2004; Neal and Wade, 2007; O'Bryant and Wade, 1999; Rosen and Wade, 2000; Wade, 2005; Winkler and Wade, 1998). During courtship, a male anole headbobs and extends a bright red throat fan, known as a dewlap. Dewlap extensions are produced by contraction of a bilateral pair of muscles located in the throat, the ceratohyoids, that cause the medially located ceratobranchial cartilages to bow out and unfold the skin under the throat (Font and Rome, 1990). Motoneurons in the caudal brainstem in the vagal (AmbX) and glossopharyngeal components of the nucleus ambiguus and ventral motor nucleus of the facial nerve (AmbIX/VIIImv) innervate the ceratohyoid muscles (Font, 1991; Wade, 1998; Wade, 2005). Following successful courtship, a male intromits one of two bilateral hemipenes. He everts it through the cloaca by contracting the transversus penis (TPN) muscles which wrap over the hemipene medially to laterally. Retraction is accomplished by the retractor penis magnus (RPM) muscles that attach to the caudal end of each hemipene, as it lies inside the tail. The copulatory motoneurons that innervate the RPM and TPN are located in the last trunk and first sacral segments (T17-S1) of the spinal cord (Holmes and Wade, 2004a; Ruiz and Wade, 2002). As in many other vertebrates (Bass and Zakon, 2005; Crews and Moore, 2005; Hull et al., 2002; Murphy and Hoffman, 2001; Panzica et al., 1996; Wilczynski et al., 2005; Wood and Swann, 2000; Yahr and Gregory, 1993), the POA and AMY (its ventromedial nucleus) mediate male sexual behaviors in the green anole (Greenberg et al., 1984; Morgantaler and Crews, 1978). Androgen receptor (AR) is expressed in these brain regions (Rosen et al., 2002), the RPM and T17-S1 motoneurons (Holmes and Wade, 2005), the ceratohyoid muscles, and about 45% of Amb X neurons, but have not been detected in AmbIX/VIIImv (Holmes and Wade, 2005; Rosen et al., 2002). In addition to controlling male sexual behaviors, adult T enhances POA and AMY soma and RPM fiber size and AR expression in the RPM (Holmes and Wade, 2004b; Neal and Wade, 2007). However, it apparently does not alter morphology or AR expression in courtship neuromuscular structures or copulatory motoneurons (Holmes and Wade, 2004b; Holmes and Wade, 2005; Neal and Wade, 2007; O'Bryant and Wade, 1999). The studies mentioned above provided some valuable information about potential mechanisms underlying behavioral variability in males. However, they also reveal that differences exist across species and between courtship and copulatory systems.

To help elucidate some fundamental processes, relationships among morphology and AR expression in the forebrain and courtship and copulatory neuromuscular systems, behavior, and circulating androgen levels were analyzed in male green anoles displaying large individual differences in reproductive behaviors. Integrating information across these characteristics

should allow us to determine the specific anatomical levels and physiological factors associated with behavioral variability.

Methods

Animals and housing

Wild-caught adult male and female green anoles were purchased from Charles Sullivan, Co. (Nashville, TN) during the breeding season, which extends from approximately April through July (Crews, 1980). They arrived on either June 1 or June 29 of 2005, and were housed in the laboratory for 9 days prior to use in this study (see below). Males were individually housed in 10-gallon glass aquaria, and stimulus females were group housed in 29-gallon aquaria. Fluorescent room lights provided long days (14 h of light). Full-spectrum and incandescent spotlights were provided above each cage, and increased cage temperatures up to 10 °C warmer than ambient, which ranged from 28 °C during the day to 19 °C at night. Relative humidity was set at 70%, facilitated by daily spraying of the cages with water. Sphagnum peat moss bedding, wooden dowels for perching, a water dish and basking rocks were provided in each aquarium. Animals were fed crickets or mealworms three times a week. Procedures were performed in accordance with Michigan State University Institutional Animal Care and Use Committee and NIH guidelines.

Behavior testing

Stimulus females were made receptive by ovariectomizing and implanting them with 5-mm-long capsules created from a slurry of Silastic sealant (Dow Corning, Midland, MI) and 2 mg estradiol benzoate (Sternaloids, Wilton, NH) that had been extracted through the tip of a 10-cm³ syringe. A novel female was introduced into the cage of each of 44 males for 15 min/day for 7 consecutive days. All behavior tests were run between 1000 and 1300 h (lights on at 800). The number of courtship displays and copulations were recorded.

Males typically show courtship behaviors, including dewlap extensions, only before mounting. However, the latency to copulation is rather variable due both to the behavior of stimulus females and the motivation of the males. In an attempt to control for these factors to some degree, a rate of dewlap extension was used for analyses. The number of dewlap extensions for each 15-min test was recorded. However, because males no longer dewlap once they begin to copulate, these values were corrected for (divided by) the fraction of the 15-min test in which they might have courted, or if the male did not copulate, the entire 15-min trial. Rates of dewlap extension were averaged for each individual across the 7 tests and used in statistical analyses.

Twenty-nine of the 44 males were selected based on the rate of dewlap extensions to obtain a continuum for correlating motivated behavior with morphology, plasma androgen levels and AR expression. Copulatory behavior was not used as a criterion because not enough variability exists; they copulate at most once per trial, in part because females exhibit an extended refractory period (Crews, 1980). Mean group differences were also assessed between the eight males that courted most frequently (studs: 17.86–31.57 average dewlap extensions per test), and the eight that courted least frequently (duds: 3.29–6.43 per test; Fig. 1A). Studs extended their dewlaps at a significantly greater rate than duds ($t=11.09$; $p<0.001$; Fig. 1B). The total number of copulations was also greater in studs, although this value did not quite reach statistical significance ($t=1.99$; $p=0.067$) due to the limited variability discussed above.

To confirm that animals from the two shipments did not differ, two-tailed, independent t -tests were performed on the rate of dewlap extension, renal sex segment size, and plasma androgen levels (all $t<0.98$; all $p>0.338$; data not shown).

Tissue collection

Approximately 24 h following the last behavior test, males were rapidly decapitated. Trunk blood was collected and plasma stored at -80°C . The brain, segments T15 through S2 of the spinal column, the ventral portion of the rostral tail containing the RPM, the throat including ceratohyoid muscles, and the kidneys were frozen in isopentane and stored at -80°C . All tissue was sectioned on a cryostat at $20\text{ }\mu\text{m}$ in 6 series, and stored at -80°C until processing for AR immunohistochemistry (as in Holmes and Wade, 2005) and histology for morphological analyses (as in Holmes and Wade, 2004b; Neal and Wade, 2007).

Radioimmunoassay

Androgen concentration from all plasma samples was measured in a single radioimmunoassay (Lovern et al., 2001; Lovorn and Wade, 2001). Briefly, the samples were thawed, mixed with $0.5\text{ ml dH}_2\text{O}$ and equilibrated overnight at 4°C with 1000 cpm of 3H-T (NET-370, 95 Ci/mmole ; NEN Life Science Products; Perkins and Elmer, Boston, MA) for individual recovery determinations. Samples were then extracted with 2 ml diethyl ether twice, dried under nitrogen, reconstituted in phosphate-buffered saline (PBS) and refrigerated overnight at 4°C . Duplicate samples were incubated with 3H-T (final working concentration of 1000 cpm) and T antibody (1:500; originally produced by Wien Laboratories, catalog #T-3003; sold by Fitzgerald, Concord, MA) overnight. Five replicate aliquots from a known concentration of a T standard were included to determine intra-assay precision. Dextran-coated charcoal (Sigma, St. Louis, MO) was added to samples to stop the reaction and remove unbound tracer. Samples were centrifuged, and the supernatant was decanted and mixed with scintillation fluid (Ultima Gold; Perkins and Elmer, Boston, MA). Bound steroid was counted on a scintillation counter (Beckman LS 6500). Samples were adjusted for individual recoveries and initial sample volume and compared to a triplicate standard curve, which ranged from 0.95 to 250 pg T . Intra-assay coefficient of variation was 12% . As the antibody cross-reacts with dihydrotestosterone (63%), total androgen concentrations are reported.

Morphological measures

One series of each tissue type was dehydrated in ethanol, cleared in xylene and stained with hematoxylin and eosin (kidney, tail and throat muscle) or thionin (brain and spinal cord tissue). Soma sizes of 20 randomly chosen neurons in the POA and AMY, 15 in AmbIX/VIIImv, 10 in AmbX, and 20 in T17-S1 were measured on each side of each animal (as in Holmes and Wade, 2004b; Neal and Wade, 2007; O'Bryant and Wade, 1999; O'Bryant and Wade, 2002) using Scion (NIH) Image software. Cross-sectional area was calculated from 25 ceratohyoid and RPM fibers per side (Holmes and Wade, 2004b; Neal and Wade, 2007; O'Bryant and Wade, 1999; O'Bryant and Wade, 2002), and in the genioglossus muscle, which was used as a control because it is in the same sections as the ceratohyoid and has a non-sexually dimorphic function, tongue protraction. TPN fibers were not analyzed because they run perpendicular to the RPM and thus cannot be measured in this preparation. Renal sex segments provide a bioassay for androgen exposure, as they increase in size as T levels rise. Thus, the heights of four epithelial cells were measured in ten tubules randomly selected from the two kidneys (Holmes and Wade, 2004b; Neal and Wade, 2007; Winkler and Wade, 1998). Averages of muscle fiber, soma and renal sex segment sizes were calculated for each individual and used in statistical analyses.

Immunohistochemistry

AR immunohistochemistry was performed as in Holmes and Wade (2005) in an alternate series of sections from those used for morphological analyses. Within each tissue type (tail, throat, brain and spinal cord), samples from all animals were run at once. Briefly, slides were warmed to room temperature and fixed in 4% paraformaldehyde for 15 min . After rinsing in 0.1 M PBS , tissue was incubated in 0.5% hydrogen peroxide for 30 min followed by 2 h in 4% normal

donkey serum in 0.1 M PBS with 0.3% Triton X-100. It was then incubated at 4 °C for 36 h in PG-21 rabbit polyclonal antibody (1.75 µg/ml for cords, throats and tails; 3.5 µg/ml for brains; Upstate Cell Signalling Solutions, Charlottesville, VA) in 0.1 M PBS with 0.3% Triton X-100 and 30% glycerol. Following a 90-min incubation in biotinylated donkey anti-rabbit secondary antibody (1:500; Jackson Laboratories, West Grove, PA), the Elite ABC kit (Vector Laboratories, Burlingame, CA) and nickel-enhanced diaminobenzidine were used to visualize AR immunoreactivity.

In the forebrain, AR+ cells were assessed in each individual in 250×100 µm² (POA) or 100×100 µm² (AMY) boxes placed in two sections from each region on both sides of the brain (total of 4 quantifications). In the POA, the short edge of each box was placed approximately 225 µm dorsal to the optic chiasm just above the suprachiasmatic nucleus and the long edge of the box was about 40 µm lateral to the third ventricle. In the AMY, the boxes were placed 50 µm dorsal to the ventral edge of the brain approximately in the center of the medial–lateral extent of the nucleus. Average counts within the boxes in each brain region were calculated for each individual. To be consistent with other studies, this value was divided by the areas used, producing an average density of AR+ cells in each individual for statistical analyses (e.g., Alexander et al., 2001a).

In the muscles, the number of AR+ cells in a 400×200 µm² (RPM) or 500×400 µm² (ceratohyoid) box was placed close to the rostrocaudal center on each side of the animals. The number of myonuclei was counted in a box of the same size in the same location of an alternate section stained with hematoxylin and eosin. Only nuclei within or touching the edge of muscle fibers were counted (Holmes and Wade, 2005). The number of AR+ nuclei was divided by the total number of myonuclei for each individual to obtain a percent for each muscle, consistent with previous work from this laboratory (Holmes and Wade, 2005).

As in Holmes and Wade (2005), the total number of AR+ nuclei was counted in T17-S1. Then, slides were placed in xylene overnight to remove coverslips and counterstained with thionin to determine the total number of motoneurons in the region. They are readily identified, as they are located almost in a line in the lateral part of the ventral horn and have characteristically large somata and dense Nissl staining (Holmes and Wade, 2004a; Holmes and Wade, 2004b, 2005; Neal and Wade, 2007; Ruiz and Wade, 2002). The number of AR+ nuclei was divided by the total number of motoneurons for each individual to calculate a percent. Because AmbIX/VIIImv does not express AR and there are very few AmbX motoneurons, less than half of which express AR (Rosen et al., 2002), these cells were not evaluated.

Statistical analysis

Correlations were conducted to determine the degree to which the number of dewlap extensions and copulations were each correlated with: (1) plasma androgen levels, (2) kidney renal sex segment height, (3) AR expression in, and (4) morphology of, the neuromuscular structures and the POA and AMY. Two-tailed unpaired *t*-tests were also performed on studs and duds to evaluate mean group differences.

Results

Plasma androgen, renal sex segments, AR expression and their relationship to behavior

Plasma androgen levels ranged from 0.18 to 8.21 ng/ml with the exception of one individual at 20.38 ng/ml. This T concentration was 4.35 standard deviations above the mean, and was a statistical outlier (Grubb's test, Sokal and Rohlf, 1981). This animal was among the 13 of those with intermediate behaviors and not categorized as either a stud or dud. Males categorized as duds had plasma androgen between 0.179 and 3.56 ng/ml. Values from studs overlapped with

some of these and ranged from 0.563 to 8.208 ng/ml. Correlations were run with and without the outlier, as we had no *a priori* reason to exclude him (he appeared healthy, behaved similarly to the other individuals, etc.). All statistical results remained the same except for the correlations of plasma androgen levels with dewlap extensions and total copulations. Both values (with and without the outlier) are reported in these cases, otherwise all analyses are reported with the outlier included, as it is more conservative.

Neither dewlap extensions ($r=0.26$; $p=0.171$) nor total copulations ($r=0.10$; $p=0.641$) correlated significantly with plasma androgen levels, unless the outlier was removed (dewlap extensions: $r=0.40$; $p=0.037$; Fig. 2A; copulations: $r=0.41$; $p=0.032$). Plasma androgen concentration did not differ between studs and duds ($t=1.88$; $p=0.081$; Fig. 2B).

As expected, plasma androgen levels and height of renal sex segment cells were positively correlated ($r=0.44$; $p=0.023$; data not shown). Renal sex segment cell height was also positively correlated with the rate of dewlap extensions ($r=0.49$; $p=0.008$; Fig. 2C) and total number of copulations ($r=0.46$; $p=0.015$; not shown), and it was greater in studs than duds ($t=2.40$; $p=0.032$; Fig. 2D).

AR expression was not correlated with plasma androgen concentration (all $r<0.24$; $p>0.244$) or renal sex segment cell height (all $r<0.35$; all $p>0.077$) in any of the tissues assessed (POA, AMY, ceratohyoid and RPM muscles, or T17-S1 motoneurons). AR+ cells in the POA and AMY were not correlated with dewlap extension (POA: $r<0.01$; $p=0.986$; AMY: $r=0.24$; $p=0.224$) or total copulations (POA: $r=0.03$; $p=0.880$; AMY: $r<0.01$; $p=0.981$). No statistically significant differences in the density of AR+ cells existed in either the POA ($t=0.04$; $p=0.971$) or AMY ($t=1.33$; $p=0.205$) between studs and duds. The percent AR+ nuclei in the ceratohyoid was not correlated with dewlap extensions ($r<0.05$; $p=0.780$), and was similar in studs and duds ($t=0.21$; $p=0.840$). No correlation existed between the percent AR+ nuclei in the RPM and total copulations ($r=0.17$; $p=0.363$), and no differences existed between studs and duds ($t=1.78$; $p=0.098$). The percent AR+ nuclei in segments T17-S1 of the spinal cord and total copulations were not correlated ($r=0.10$; $p=0.686$), and the percent AR+ nuclei did not differ between studs and duds ($t=0.65$; $p=0.525$).

Morphology and behavior

Soma size in the POA was not correlated with the rate of dewlap extensions ($r=0.32$; $p=0.098$) or total number of copulations ($r=0.03$; $p=0.773$). However, average soma size in the AMY was positively correlated with dewlap extensions ($r=0.39$; $p=0.047$; Fig. 3), although not with copulations ($r=0.17$; $p=0.358$). No statistically significant difference existed in either POA ($t=-0.31$; $p=0.763$) or AMY ($t=-0.64$; $p=0.531$) soma size between studs and duds.

Ceratohyoid fiber size was positively correlated with dewlap extensions ($r=0.44$; $p=0.017$; Fig. 4A) and was significantly larger in studs than duds ($t=4.03$; $p=0.001$; Figs. 4B and 5). Fiber size in the control, genioglossus, muscle was not correlated with dewlap extensions ($r=0.30$; $p=0.108$) and was equivalent in studs and duds ($t=1.89$; $p=0.080$). AmbIX/VIIImv and AmbX soma sizes were not correlated with expression of either behavior (all $r<0.14$; all $p>0.414$). No statistically significant differences existed between studs and duds in AmbIX/VIIImv ($t=0.36$; $p=0.725$) or AmbX ($t=0.79$; $p=0.443$) soma size.

Total number of copulations and RPM fiber size were not correlated ($r<0.05$; $p=0.747$), and no statistical differences in RPM fiber size existed between studs and duds ($t=0.33$; $p=0.744$). T17-S1 motoneuron soma size ($r<0.05$; $p=0.739$) was not correlated with number of copulations, and T17-S1 motoneuron soma size did not differ between studs and duds ($t=1.00$; $p=0.333$).

Discussion

Summary

Significant positive relationships between behavior and morphology existed in the ceratohyoid muscle and AMY, suggesting that the size of cells in these structures could underlie individual variations in courtship behaviors in the male green anole. However, at this point it is not clear whether a causal relationship between behavior and morphology exists, and if so, the direction in which it lies. Plasma androgen concentration and the height of renal sex segment cells were also associated with increased behavior. However, the kidney data (which is commonly used in lizards as a bioassay for the effectiveness of androgen treatment, Holmes and Wade, 2004b, 2005; Neal and Wade, 2007; O'Bryant and Wade, 1999; Winkler and Wade, 1998) showed a stronger relationship to behavior than the plasma hormone levels. This pattern is consistent with the idea that an individual's physiological response to T may play a larger role in mediating behavior than the hormone concentration itself. Further, as androgen receptor protein levels (at least as quantified in the present study) were not associated with behavioral expression, it appears that they are not the critical factor in conferring the variability in the response. It is also possible, although we believe less likely, that renal sex segment morphology is more directly associated with behavioral variability than circulating androgen levels because behavior and morphological effects reflect androgen integrated over a period of time, whereas plasma hormone concentration represents a single "snapshot". These ideas will be explored more fully in the sections below.

Morphology and behavior

Comparing the results from the studies investigating relationships between form and function in green anole reproductive systems allows us to generate some hypotheses regarding mechanisms. For example, the sizes of motoneurons involved in dewlap extension and copulation are not associated with behavior under any circumstance we have investigated. These measures were not correlated with behavior in the present study, and previous experiments (Holmes and Wade, 2004b; Neal and Wade, 2007; O'Bryant and Wade, 1999) have shown that they are not altered by T treatment, seasonal environmental cues or female exposure. Thus, all of the data collected so far suggest that the size of these motoneurons does not covary with or appear to produce variation in behavioral function.

A different situation exists for the target muscles, in which morphology is in some cases related to behavior. The copulatory muscles (e.g., RPM) are present only in males (Holmes and Wade, 2004a; Ruiz and Wade, 2002), and the size of their fibers increases in castrated animals treated with T (and more so in the breeding than non-breeding season, Holmes and Wade, 2004b; Neal and Wade, 2007). Because these relatively high doses of T increase AR expression in the RPM (Holmes and Wade, 2005), upregulation of this protein may play a role in the structural change and/or the activation of copulatory behavior induced by T. However, normal variations in T levels, either across gonadally intact individuals within a season (as in the present study) or between seasons (as in previous experiments, Holmes and Wade, 2004b), do not appear sufficient to induce RPM plasticity. Similarly, the present data comparing duds to studs, which copulated almost 10 times as often, and previous results comparing individuals exposed to females and those which were not (Neal and Wade, 2007), all suggest that the level of RPM use is not associated with the size of its fibers. It should be noted, however, that it is possible that insufficient variability in copulatory behavior could explain the lack of a relationship between size and function, as males copulated at most once during each test, and not all males copulated each day.

In contrast to the RPM, the size of ceratohyoid fibers has not been affected by any of the manipulations we have used in previous studies that induce dramatically different frequencies

of courtship behavior, including the large difference in hormone levels between castrated animals implanted with T and those given control capsules (Neal and Wade, 2007; O'Bryant and Wade, 1999). Yet, significant correlations between morphology and level of function existed in unmanipulated, gonadally intact animals during the breeding season in the present study. These results are specific to this muscle controlling dewlap extension; neither the copulatory RPM nor the genioglossus, which was evaluated as a control near the ceratohyoid, showed this relationship. This association between 'natural' variability in unmanipulated, gonadally intact animals is similar to the large sex difference in ceratohyoid fiber size. They are substantially (and significantly) larger in males, who use their dewlaps approximately 7 times as often, than females (Jenssen et al., 2000). One possibility is that fiber size is not plastic in adulthood; that once developed it permanently confers a level of relative behavior function. Males do occasionally extend their dewlaps in the non-breeding season (Jenssen et al., 1996; Lovern et al., 2004; Neal and Wade, 2007; O'Bryant and Wade, 1999), and we have hypothesized that animals must therefore maintain fiber size during this time. It would be useful at this point to determine whether this limited dewlap use in the non-breeding season is also correlated with ceratohyoid fiber size. Similarly, it would be interesting to know whether high performers in the breeding season are also high performers compared to others in the non-breeding season. While unlikely, it is of course possible that fiber size is plastic in adulthood, but that the duration of female exposure in this experiment was not long enough to induce a difference in the ceratohyoid.

Results on soma size in the AMY parallel those on ceratohyoid fiber size to some degree, in that a significant correlation between cell size and rate of dewlap extension existed in the present study. However, unlike the muscle, a significant difference was not detected between studs and duds with a *t*-test in the AMY. It is difficult to reconcile the data from the two types of analyses of this brain region. However, on average studs did have slightly larger (6%) soma sizes in the AMY than duds. The correlation of soma size with the rate of dewlap extension detected in the AMY, but not the POA, suggests a relatively specific relationship between structure and function. The AMY integrates sensory, visceral and cognitive information and relays it to the POA for the expression of male reproductive behaviors in some mammalian, reptilian and avian species (Goodson, 2005; Greenberg et al., 1984; Kondo and Arai, 1995; Kondo and Yamanouchi, 1995; Newman, 1999; Paredes, 2003; Wood, 1997; Wood and Coolen, 1997; Wood and Swann, 2000). Although the POA and AMY are both involved in sexual motivation and behavior (Hull and Dominguez, 2006; Wood and Coolen, 1997), it has been suggested that the POA may be more important for motor aspects of sexual behavior (Wood, 1997). Therefore, subtle differences in morphology of a brain region might lead to the differences observed in individual variations in behavior.

Similar to the present study, differences exist in neuromuscular morphology between groups of male sonic fish displaying various levels of courtship behavior; muscle mass and motoneuron soma size are larger in males that vocalize than those that do not (Bass and Baker, 1990; Bass and Marchaterre, 1989; Bass and Zakon, 2005; Brantley et al., 1993). In contrast, rat copulatory neuromuscular structures can be diminished in intact males implanted with sub-physiological levels of T that copulate frequently compared to non-copulating males (Breedlove, 1997). These results, however, were not replicated by Raouf et al. (2000), who paradoxically found reduced T levels following female exposure and sexual activity in castrated, T-treated males.

Also similar to the present study, in some other species behavioral variability is related to within-sex differences in the morphology of reproductively relevant forebrain structures. For example, in some songbirds, natural variations in the volumes of song control regions correlate with the amount of song produced by an individual (Bernard et al., 1996). In male rats and rams, AMY (and POA, unlike anoles) volume and neuron soma sizes are larger in groups of

males exhibiting high compared to low sexual activity (Alexander et al., 2001b; Prince et al., 1998).

Role of androgen and its receptors

While T is critical to masculine behavior in the green anole, and in fact is more potent in activating them than estradiol and/or dihydrotestosterone (Adkins and Schlesinger, 1979; Crews et al., 1978; Lindzey and Crews, 1986; Mason and Adkins, 1976; Noble and Greenberg, 1941; Wade et al., 1993; Winkler and Wade, 1998), the present data suggest that physiological response of the renal sex segments appears to be a more accurate indicator of individual variations in male sexual behavior than the circulating level of the hormone itself. That is, renal sex segment cell height was positively correlated with the display of reproductive behaviors and was greater in groups of studs than duds. In contrast, statistical significance was only reached for correlations between plasma androgen concentration and courtship and copulatory behaviors when the outlier was not included in the analyses, and the hormone level did not differ significantly between high and low sexual performers. Similarly, the proportion of behavioral variability explained by the kidney data ($r=0.49$) is substantially higher than that for plasma androgen ($r=0.14$ without the outlier, 0.26 with him). Studies in the male guinea pig, ram and whiptail lizard also indicate that differences in sexual behavior are not the result of basal levels of plasma androgen (Alexander et al., 1999; Crews, 1998; Harding and Feder, 1976; Lindzey and Crews, 1992; Perkins et al., 1992).

One might be concerned that the behavioral testing had an impact on the T levels detected in the present study, as has occurred in rats and rams (Alexander et al., 1999; Harding and Feder, 1976; Perkins et al., 1992), rather than the reverse. However, to diminish the likelihood that differences in T levels could be explained by social interaction, tissue was collected 1 day following the last behavioral test in the present study. Alternatively, one might consider that the individual variations we detected in behavior were due to differences in the length of time males were exposed to T. However, this idea is unlikely for at least two reasons. First, the testes begin to recrudescence in male green anoles around November, resulting in increased T levels (Crews, 1980). By the time courtship behavior begins in April (Crews, 1980; Lovern et al., 2004), they have been exposed to at least some T for many months and for even longer by the time we tested them mid-summer. Second, the fact that behavior was not increased in animals from the second compared to first shipment we received also documents that exposure to T for an additional month does not increase behavior. Still, it is possible that T levels might relate more strongly to behavior than detected in the present study. The values obtained reflect only the plasma concentration at one time point. In contrast, morphological and functional responses of regions of the central nervous system, muscles and kidney all likely reflect an integration of hormonal exposure after a much longer period.

Regardless, AR protein was not related to behavioral variability in any of the tissues examined in the present study. In rats, *estrogen* receptor immunoreactivity is greater in the POA in groups of high compared to low sexual performers (Clark et al., 1985), and in rams, more estrogen binding occurs in the POA and AMY in studs than duds (Alexander et al., 1993), even with similar plasma T and estrogen levels. Also, aromatase activity in non-copulating rats is lower than rats that copulate frequently (Portillo et al., 2007). While conceivable, it seems unlikely that increased estrogen receptor expression or aromatase activity enhances sexual behavior in male green anoles, as hormone manipulation studies indicate no effect of estradiol on masculine sexual behavior in this species (Adkins and Schlesinger, 1979; Crews et al., 1978; Lindzey and Crews, 1986; Mason and Adkins, 1976; Noble and Greenberg, 1941; Wade et al., 1993; Winkler and Wade, 1998). However, it is possible that individual variations in male behavior could be related to the number of AR per cell or differences in binding affinity, which could not be assessed with the immunohisto-chemical technique used in the present study. Or other

related mechanisms independent of steroid receptors themselves, such as the expression of steroid receptor co-activators might be important (MacLean et al., 1997; O'Bryant and Jordan, 2005). Future studies should examine these possibilities.

Conclusions

Results obtained from green anoles to date converge on the ideas that the size of fibers in the muscle critical for dewlap extension and cells in the AMY are specifically associated with the level of behavioral display. T activates sexual behaviors in this species and can enhance the size of somas in the POA and AMY, as well as copulatory muscle fiber size (Holmes and Wade, 2004b; Neal and Wade, 2007; O'Bryant and Wade, 2002). It appears that some factor(s) intrinsic to the tissues other than density of nuclei expressing AR, is (are) related to the level of behavior displayed, more so than the plasma androgen concentration. The green anole genome is currently being sequenced, and as molecular tools become available, we will be in an excellent position to identify critical mechanisms.

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Fig. 1.

Values represent the average number of dewlap extensions per test. Individuals only courted up to the time they began to copulate. Therefore, to assess the relative rates of courtship over this 15-min period, the number of dewlap extensions was divided by the fraction of the test prior to copulation (by the amount of time during which the animal might have courted). Each dot in panel A represents the average for each male exposed to a different female over seven trials. The mean group differences between the eight studs and eight duds is depicted in panel B (means+standard errors; * $p < 0.0001$).

Fig. 2.

Relationships between behavior and plasma androgen (A and B) or renal sex segment height (C and D). Panels A and C depict correlations of the two physiological measures with dewlap extension rate. Panels B and D indicate comparisons between high- and low-intensity courters. The open circle in panel A indicates the outlier (means+standard errors; N.S.=not statistically significant).

Fig. 3.
Average amygdala (AMY) soma size was positively correlated with the rate of dewlap extensions.

Fig. 4. Relationships between muscle fiber size and dewlap extension rate. Panel A depicts a significant correlation ($p=0.017$), and panel B documents the mean group difference between studs and duds (means+standard errors; $p=0.001$).

Fig. 5. Photomicrographs of cross-sections through ceratohyoid muscle fibers in a stud (top) and dud (bottom). Scale bar=50 μ m.