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ADULT PLASTICITY OF COURTSHIP AND COPULATORY BEHAVIORS AND ASSOCIATED MORPHOLOGY IN THE MALE GREEN ANOLE (*ANOLIS CAROLINENSIS*)

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Jennifer Kathleen Neal

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ADULT PLASTICITY OF COURTSHIP AND COPULATORY BEHAVIORS AND ASSOCIATED MORPHOLOGY IN THE MALE GREEN ANOLE (*ANOLIS CAROLINENSIS*)

By

Jennifer Kathleen Neal

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ABSTRACT

ADULT PLASTICITY OF COURTSHIP AND COPULATORY BEHAVIORS AND ASSOCIATED MORPHOLOGY IN THE MALE GREEN ANOLE (*ANOLIS CAROLINENSIS*)

By

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Studying relationships between behavior and morphology reveals mechanisms underlying adult plasticity. The display of reproductive behaviors requires the coordination of structures from forebrain regions involved in courtship and copulation, to motoneurons and muscles necessary for the execution of these behaviors, thus providing a system in which to study relationships between behavior and morphology and mechanisms underlying adult plasticity. The degree to which the expression of reproductive behaviors can be modulated varies depending on specific cues the animal is exposed to, including hormonal and environmental factors. These relationships can be studied within the same individuals in one species, the green anole lizard (*Anolis carolinensis*). This species breeds seasonally, and neuromuscular systems controlling courtship and copulation respond differently to seasonal and hormonal cues. The following studies increased the understanding of the underlying mechanisms controlling adult structural and behavioral plasticity, as well as the potential for inducing plasticity.

DEDICATION

I want to dedicate this dissertation to my husband, Chisom, whose steadfast love and support kept me from losing my mind. I also thank my family for their continuous encouragement.

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CHAPTER 1: INTRODUCTION

An organism's survival depends on its ability to adapt to changes in its internal and external environment. By examining naturally occurring and experimentally created differences in behavior, the mechanisms regulating and limiting adult morphological and behavioral plasticity can be uncovered. Reproductive systems offer advantages for studying structure-function relationships because the behaviors are stereotypical and easily manipulated in the lab, and in many cases the circuits regulating them have been defined. The coordination of motivational forebrain areas, motoneurons, and the muscles they innervate is necessary for the optimal display of male sexual behavior. The following work investigated structural and functional changes at these levels.

Hormonal and seasonal regulation of male reproductive behavior

Male reproduction involves species-specific courtship and copulatory behaviors. In some species, courtship displays are characterized by vocalizations or visual displays. Copulation in some vertebrate groups, including mammals and reptiles, involves intromission of a copulatory organ (Hull, et al., 2002; Wade, 2005).

Testosterone (T) and/or its metabolites, estrogen and dihydrotestosterone, are vital to the expression of male sexual behavior in many mammalian, avian and reptilian species. Male sexual behavior is reduced or eliminated by castration and can be restored by administration of T (Paredes, 2003; Romeo, et al., 2001; Sakata, et al., 2003; Wood, 1996; Wood and Swann, 2000). T and dihydrotestosterone bind AR, and estrogen activates estrogen receptors to facilitate male sexual behavior in some rodents (Cooke, 2005; Cooke, et al., 1999; Hamson, et al., 2004; McGinnis and Dreifuss, 1989; Paredes,

2003; Simerly, et al., 1990). However, estrogen and DHT are not necessary for the expression of male sexual behavior in all animals. T alone can regulate the expression of male sexual behavior in lizards (Crews, et al., 1978; Winkler and Wade, 1998).

Seasonal breeders exhibit naturally occurring behavioral plasticity. They court and copulate when environmental conditions are most suitable to maximize offspring survival and cease the display of sexual behaviors when it would be energetically costly (Prendergast, et al., 2002). Fluctuating levels of T, due to gonadal regression and recrudescence between season, are associated with changes in male sexual behavior (Ball, et al., 2004). However, T is not completely responsible for changes in behavior between season. Differences in behavior may also be mediated by other hormones, including melatonin, and external factors such as photoperiod and temperature (Prendergast et al., 2002).

Forebrain control of male sexual behavior

In many mammals, birds, fish, amphibians and reptiles, the preoptic area (POA) and amygdala (AMY) are involved in the production of male sexual behavior (Ball and Balthazart, 2004; Bass and Zakon, 2005; Crews and Moore, 2005; Hull et al., 2002; Kondo and Arai, 1995; Murphy and Hoffman, 2001; Panzica, et al., 1996; Wilczynski, et al., 2005; Wood and Swann, 2000; Yahr and Gregory, 1993). Behavioral adaptations are often associated with morphological changes in forebrain structures, including alterations in volume of brain regions, neuronal number or spacing, dendritic arborization, or neuron soma size. Plasticity of the adult brain, and its effects on behavior, has been studied in diverse species and will be discussed below.

The morphology of brain regions involved in male sexual behavior change between season in some songbirds, mammals and reptiles (Ball et al., 2004; Caro, et al., 2005; Cooke, 2005; Cooke, et al., 2003; Romeo and Sisk, 2001; Wade, 2005). For example, fluctuations in songbird singing behavior between seasons correlate with changes in neural attributes of song production areas of the brain, including changes in volume, neuron soma size, dendritic morphology, neuron survival, or neuronal density. These changes can be due to additive effects of seasonal increases in T and song production (Alvarez-Borda and Nottebohm, 2002; Brenowitz, 1997; Brenowitz and Beecher, 2005; Li, et al., 2000; Nottebohm, et al., 1986; Rasika, et al., 1994; Thompson and Brenowitz, 2005; Tramontin and Brenowitz, 2000; Tramontin, et al., 1999). T can enhance the volume of the POA in parallel to increases in male sexual behavior in other seasonally breeding birds and some reptiles (Crews, et al., 1993; Panzica et al., 1996; Thompson and Adkins-Regan, 1994; Wade and Crews, 1991; Wade, et al., 1993).

Expression of immediate-early genes, markers of neuronal activity, also correlates with increases in male sexual behavior in some species of mammals and birds. For example, courtship and copulation increases immediate early gene expression in the POA and AMY of rodents and songbirds, as well as in avian song production areas (Heeb and Yahr, 1996; Kollack-Walker and Newman, 1995; Kollack-Walker and Newman, 1997; Mello, 2002; Schwab, et al., 2004), and in AR-expressing cells of the POA in rodents (Greco, et al., 1998). Additionally, T and season can interact to produce changes in neuronal activity. In starlings, immediate early gene expression in the POA and song nuclei correlates with behavior during the breeding season (BS) but not non-breeding

season (NBS) in response to female presence (Heimovics and Riters, 2005; Heimovics and Riters, 2006).

Neuromuscular control of male sexual behavior

Because a direct link exists between neuromuscular activity and behavior (Breedlove, et al., 2002), the study of adult behavioral and morphological plasticity is straightforward and practical in courtship and copulatory neuromuscular systems. Neuromuscular plasticity has been studied in structures required for courtship vocalizations in birds, frogs and fish (Brahic and Kelley, 2003; Knapp, et al., 1999; Tobias and Kelley, 1987; Tramontin and Brenowitz, 2000). The study of male copulatory neuromuscular systems has largely been confined to the spinal nucleus of the bulbocavernosus (SNB) motoneurons and the penile muscles they innervate in mammals (Breedlove and Arnold, 1980; Breedlove and Arnold, 1981; Breedlove and Arnold, 1983). In all of these systems, adult T enhances neuromuscular morphology, and AR is expressed in the courtship and copulatory neuromuscular structures of some birds, mammals, frogs and fish (Arnold and Breedlove, 1985; Bass and Marchaterre, 1989; Bass and Zakon, 2005; Breedlove and Arnold, 1980; Breedlove and Arnold, 1981; Breedlove and Arnold, 1983; Cooke, et al., 1998; Forger, et al., 1992; Forger, et al., 1996; Gurney, 1981; Gurney, 1982; Kay, et al., 1999; Kelley, 2004; Kelley, et al., 1988; Park, et al., 2002; Potter, et al., 2005; Rand and Breedlove, 1995; Wade and Buhlman, 2000; Wetzel and Kelley, 1983). Seasonally induced changes in motoneuron or muscle morphology can be the result of fluctuating levels of circulating T in seasonal breeders (Forger and Breedlove, 1987; Hegstrom, et al., 2002).

Neuromuscular activity and androgens interact to affect morphology. For example, the frog courtship neuromuscular system is sensitive to both neuromuscular activity and androgens, and elimination of either one results in decreased muscle fiber size (Tobias, et al., 1993). Increases in neuromuscular activity are correlated with increases in neuromuscular morphology in some fish, songbirds and mammals. For example, in a species of vocalizing fish with two male morphs, sonic muscle mass and motoneuron soma size is greater in males that vocalize than males that do not (Bass and Baker, 1990). In some songbird species, sexually dimorphic singing behavior, in which males sing and females do not, is correlated with sexually dimorphic neuromuscular structures. Similarly, monomorphic singing behavior, in which males and females sing equally, is correlated with monomorphic neuromuscular morphology (Brenowitz, 1997; DeVoogd, et al., 1995).

Individual variations in male reproductive behavior and morphology

The studies described above have generally investigated group differences in male sexual behavior and morphology. However, examining individual differences more directly may reveal the degree to which behavior and morphology are related. Investigations of individual variation in sexual behavior have focused on differences in circulating hormones, morphology of brain areas involved in male sexual behavior and steroid receptor expression. Although T levels are higher in high male sexual performers than in low sexual performers immediately following female exposure in guinea pigs and rams, basal T does not appear to explain variations in male sexual behavior (Alexander, et al., 1999; Grunt and Young, 1952; Harding and Feder, 1976; Lindzey and Crews,

1992; Perkins, et al., 1992). Differences in plasma T after contact with females may indicate individual variations in the male's perception of the sexual situation, disparity in sensitivity to androgens or an inability to exhibit sexual behavior (Crews, 1998). Individual variations in behavior may also be the result of differences in morphology of reproductively relevant brain structures. In male rats and rams, POA and AMY volume and neuron soma sizes are larger in males exhibiting high compared to low sexual activity (Alexander, et al., 2001b; Prince, et al., 1998). In some songbirds, natural variations in song control volumes correlate with the amount of song produced (Bernard, et al., 1996). Steroid receptor expression levels may also be involved in creating individual variations in male sexual behavior. In rams, more estrogen receptors are occupied by estrogen in high than low sexual performers, even in the presence of similar plasma T and estrogen levels (Alexander, et al., 1993). Similarly, estrogen receptor expression is reduced in the POA of low compared to high sexual performing rats (Clark, et al., 1985). Individual variations in male behavior may also result from differences in courtship and/or copulatory neuromuscular structures. For example, neuromuscular morphology positively correlates with levels of courtship displays in sonic fish. Muscle mass and motoneuron soma size is larger in males that vocalize than males that do not vocalize (Bass and Baker, 1990; Bass and Marchaterre, 1989; Bass and Zakon, 2005; Brantley, et al., 1993). In contrast, male rats that copulate frequently have decreased SNB muscle mass and motoneuron soma size than males that copulate infrequently (Breedlove, 1997; Raouf, et al., 2000).

The male green anole reproductive behavior and morphology

A species that reliably displays male sexual behavior under conditions in which it can be manipulated and quantified, is needed to study adult plasticity in neural and muscular systems and the underlying mechanisms involved (Hull et al., 2002). An integrated picture of how hormones, season and behavior affect morphology at multiple levels (forebrain, motoneurons and muscles) is necessary for understanding adult plasticity in reproductive systems. The green anole provides these and other advantages for studying mechanisms of adult plasticity.

While previous studies of structure-function relationships in model systems have examined courtship and copulatory systems in separate vertebrate groups (see above), green anoles possess both courtship and copulatory neuromuscular systems (described below). Studying them within the same individuals allows for numerous variables to be kept constant in ways not possible across species. Typically, neuromuscular systems and motivational forebrain areas are also examined separately (e.g., Arnold and Breedlove, 1985; Breedlove and Arnold, 1980; Breedlove and Arnold, 1981; Breedlove and Arnold, 1983; Cooke, 2005; Cooke et al., 1999). The following experiments investigated both motivational forebrain regions and neuromuscular systems in the same studies, allowing for direct comparisons of mechanisms involved: from the motivation to produce the behavior (POA and AMY) to the physical production of both courtship and copulation (neuromuscular).

The green anole is a seasonal breeder, and males exhibit a characteristic suite of sexual behaviors. In courtship and aggressive encounters, male anoles bob their heads and extend bright red throat fans, known as dewlaps. Dewlap extensions occur far more often in the BS than NBS, and copulation only occurs during the BS (Greenberg and

Nobel, 1944; Jenssen, et al., 2000). If courtship is successful, the male will copulate with the female by intromitting one of two bilateral hemipenes. T increases courtship and copulatory behaviors during the BS, but only courtship behaviors during the NBS (Lovern, et al., 2004b; O'Bryant and Wade, 1999; Wade, 2005).

The ventromedial nucleus of the AMY and dorsal POA facilitate male courtship and copulatory behavior in lizards. Lesions to the POA and AMY reduce courtship displays and eliminate copulation (Greenberg, et al., 1984; Morgantaler and Crews, 1978). These regions are sensitive to seasonal manipulations. Soma sizes of POA and AMY neurons are smaller during the NBS than BS. However, T administration during the NBS does not increase soma size, suggesting that the POA and AMY may either be less sensitive to T during the NBS, or that changes in soma size are not the direct result of seasonal fluctuations in T (O'Bryant and Wade, 2002). AR are present in the POA and AMY (Rosen, et al., 2002), but it is currently unknown whether hormonal, seasonal or social factors affect expression.

To unfold the dewlap, males contract a set of muscles, the ceratohyoids, located on each side of the throat. This contraction causes the medially located ceratobranchial cartilages to bow out, unfolding the dewlap. The ceratohyoid muscles are innervated by 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/VIImv) (Wade, 1998). Adult males have larger muscle fiber size and motoneuron soma sizes than females. AR is expressed in the ceratohyoid muscles and Amb X, but not AmbIX/VIImv. Neither T nor seasonal manipulations have altered AR

expression in courtship neuromuscular structures (Holmes and Wade, 2005b). They also have not affected motoneuron soma or muscle fiber size (O'Bryant and Wade, 1999).

The anole copulatory system consists of motoneurons located in the last trunk and first sacral segments (T17-S1) of the spinal cord that innervate the retractor penis magnus (RPM) and transversus penis (TPN) muscles, which retract and evert the hemipenes respectively. Females do not have hemipenes or the associated structures regulating them (Holmes and Wade, 2004a; Ruiz and Wade, 2002). AR is expressed in the hemipenes, RPM and T17-S1 motoneurons. In intact males, AR expression does not change between season. T increases AR expression in the hemipenes and RPM, but not the motoneurons, regardless of season (Holmes and Wade, 2005b). T increases hemipene and RPM fiber sizes and the effect is greater during the BS than NBS. No effect of T or season exists on T17-S1 motoneuron soma size (Holmes and Wade, 2004b).

While the effects have been quite clean and reliable, previous work on the anole (described above) investigated the forebrain and two neuromuscular systems independently, with different individuals in different years. So, the potential plasticity within these systems could not be certain until all levels of the nervous system were considered simultaneously within the same individuals. Previous studies investigated hormonal and seasonal effects on forebrain and neuromuscular morphology in the absence of female social interactions. However, social interactions might also induce changes in reproductive structures, either by stimulating increases in their use, or less directly through other social cues. Thus, in Chapter 2, the effects of hormonal, seasonal and social cues on anole courtship and copulatory behavior and morphology were examined in males exposed to T or vehicle control under either BS or NBS temperatures

and photoperiods, as well as to one of three levels of exposure to females to reveal the degree of plasticity possible.

Both experimental manipulation and utilizing individuals with naturally occurring differences in behavioral function can provide important information. Previous experiments have examined mean group differences in courtship and copulatory morphology in gonadally intact males between seasons (e.g., Holmes and Wade, 2004b; O'Bryant and Wade, 2002), but they have not investigated relationships between behavior and potential underlying mechanisms. In naturally occurring populations of male anoles individual variations in reproductive behavior exist; some court and copulate frequently (studs) and others do not (duds). Therefore, in Chapter 3, potential mechanisms underlying behavioral variability were investigated. Morphology of the POA and AMY and courtship and copulatory neuromuscular structures, circulating plasma androgen levels and AR expression were examined in males exhibiting individual variations in behavior to reveal natural relationships among behavior, morphology and AR, but not the direction of these relationships.

Although it is well documented that reproductive behavior is associated with increased neuronal activity in the POA and AMY in rodents, songbirds and fish (Greco, et al., 1996; Heeb and Yahr, 1996; Kollack-Walker and Newman, 1995; Kollack-Walker and Newman, 1997; Mello, 2002; Schaefer and Zakon, 1996; Schwab et al., 2004; Shimura, et al., 1994), and is influenced by T, season and social cues in some birds and rodents (Heimovics and Riters, 2005; Heimovics and Riters, 2006; Mello, 2002; Schwab et al., 2004), immediate early gene expression has not been investigated in the male green anole forebrain. Therefore, to determine whether sexual behavior in male anoles was

associated with changes in neuronal activity, the effects of T, season and female social contact on c-fos expression in the POA and AMY were examined in Chapter 4.

CHAPTER 2

Neal J.K. and Wade J. (2007) Courtship and copulation in the adult male green anole: Effects of season, hormone and female contact on reproductive behavior and morphology. Behavioural Brain Research. 177(2):177-185.

CHAPTER 2: COURTSHIP AND COPULATION IN THE ADULT MALE GREEN ANOLE: EFFECTS OF SEASON, HORMONE AND FEMALE CONTACT ON REPRODUCTIVE BEHAVIOR AND MORPHOLOGY

Abstract

Interactions among reproductive season, testosterone (T) and female presence were investigated on the structure and function of forebrain and neuromuscular systems controlling courtship and copulation in the green anole lizard. Under breeding (BS) or non-breeding (NBS) environmental conditions, male green anoles were implanted with either T or blank capsules and exposed to one of three female stimulus conditions: physical, visual or no female contact. T and at least visual exposure to females increased courtship displays (extension of a throat fan, or dewlap), and these effects were greater during the BS than NBS. T also facilitated copulation, and did so to a greater extent in the BS. The hormone increased soma size in the preoptic area (POA) and amygdala (AMY), and in the AMY the effects were greater in the BS than NBS. Cross-sectional areas of copulatory organs and associated muscle fibers were enhanced by T, and more so in the BS than NBS. However, no effects on morphology of dewlap motoneurons or muscles or copulatory motoneurons were detected. Thus, (1) changes in behavior and neural and/or muscular morphology are not always parallel and (2) differences in responsiveness to T exist across seasons and among tissues.

Introduction

Behavioral plasticity is crucial for an animal to adapt to changing environmental influences, and it is frequently accompanied by modifications in the morphology of underlying structures. These types of effects are often seen in reproductive systems of seasonally breeding vertebrates (Ball and Balthazart, 2004; Ball, et al., 2002; Brenowitz, 2004; Hegstrom and Breedlove, 1999; Jacobs, 1996; Lovern et al., 2004b; Prendergast et al., 2002; Tramontin and Brenowitz, 2000). Sexual behaviors can include courtship exhibited by males, such as vocalizations or visual displays, and copulatory behaviors that in some cases involve intromission of a penis by the male (Hull et al., 2002; Wade, 2005). While the specific behaviors vary widely across species, testosterone (T) and/or its metabolites estradiol and dihydrotestosterone are vital to the expression of male sexual behaviors across many mammals, birds and reptiles (Cooke, 2005; Cooke et al., 1999; McGinnis and Dreifuss, 1989; Paredes, 2003; Romeo et al., 2001; Sakata et al., 2003; Simerly et al., 1990; Wood, 1996; Wood and Swann, 2000).

The expression of male sexual behavior requires the coordination of forebrain regions and neuromuscular circuits (Breedlove et al., 2002). In diverse vertebrates, the preoptic area (POA) and amygdala (AMY) are necessary for the production of male-specific courtship and copulatory behaviors (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). Motoneurons located in the brainstem or spinal cord innervate muscles required for courtship and copulatory behaviors. For example, muscles surrounding the syrinx in songbirds (Vicario, 1991), swim bladder in some fishes (Bass, et al., 1994), and larynx in frogs (Kelley, 2004) are

innervated by caudal brainstem motor nuclei and facilitate vocalizations used by males to court females. Similarly, mammalian copulation involves muscles surrounding the base of the penis that are innervated by motoneurons in the caudal spinal cord (Breedlove and Arnold, 1980). Many of these structures exhibit plasticity that parallel seasonal or T-induced changes in behavior (Brantley et al., 1993; Breedlove and Arnold, 1981; Breedlove and Arnold, 1983; Connaughton and Taylor, 1995; Kelley, 1986; Panzica et al., 1996; Potter et al., 2005; Thompson and Adkins-Regan, 1994; Tramontin and Brenowitz, 2000; Wetzel and Kelley, 1983), and they have provided valuable insights about relationships between behavior and morphology. However, full appreciation for key mechanisms may be diminished by studying courtship and copulatory neuromuscular circuits in separate vertebrate groups. To some extent this has been done for good reason; most frogs, fish and birds (whose courtship systems have been exceptional models) do not have penises, and mammals (whose copulatory systems have been extensively studied) do not usually have courtship displays amenable to investigation. It is also rare that forebrain and neuromuscular morphology are investigated in the same experiments.

Green anole lizards (*Anolis carolinensis*) offer a unique opportunity to investigate in the same individuals the multiple levels of the nervous system required to coordinate reproduction, thus allowing direct comparisons among them. This species breeds seasonally, and males exhibit a characteristic suite of sexual behaviors that has been extensively studied in the field and lab (Crews and Moore, 2005). In a courtship encounter, a male anole bobs his head and extends a bright red throat fan, known as a dewlap. If courtship is successful, the male will copulate with the female by intromitting

one of two bilateral hemipenes, which normally lay inside the ventral portion of the tail. In unmanipulated animals, dewlap extensions occur far more often in the breeding (BS) than non-breeding (NBS) season, and copulation only occurs during the BS (Greenberg and Nobel, 1944; Jenssen et al., 2000). A seasonal increase in T, rather than its metabolites, is the primary regulator of male sexual behavior (Crews et al., 1978; Lovern et al., 2004b; Lovern, et al., 2001; Rosen and Wade, 2000; Winkler and Wade, 1998).

As in other vertebrates, the POA and AMY (its ventromedial nucleus) facilitate male sexual behaviors in green anoles (Greenberg et al., 1984; Morgantaler and Crews, 1978). The neuromuscular components required for courtship include a bilateral pair of muscles located in the throat, the ceratohyoids, which cause the medially located ceratobranchial cartilages to bow out and unfold the dewlap (Font and Rome, 1990). These muscles are innervated by motoneurons in the caudal brainstem in the vagal component of nucleus ambiguus (AmbX) and the area containing the glossopharyngeal portion of nucleus ambiguus and the ventral motor nucleus of the facial nerve (AmbIX/VIImv) (Font, 1991; Wade, 1998; Wade, 2005). To copulate, males evert one of their hemipenes through the cloaca by contracting the transversus penis muscle (TPN) that wraps over it medially to laterally. They retract the hemipene by contracting the retractor penis magnus muscle (RPM) which attaches to the caudal end of the organ as it lies inside the tail. The motoneurons innervating the muscles controlling penis movement are located in the last trunk and first sacral segments (T17-S1) of the spinal cord (Holmes and Wade, 2004a; Ruiz and Wade, 2002; Wade, 2005).

Previous studies of the anole POA and AMY and the courtship and copulatory neuromuscular structures have revealed differences in plasticity in regard to T and

season. In intact animals, POA and AMY soma sizes are smaller during the NBS than BS, and T administration during the NBS has no effect on this measure (O'Bryant and Wade, 2002). Although courtship behavior dramatically decreases during the NBS compared to the BS and in castrated compared to T-treated males, the size of dewlap muscle fibers and motoneuron somas appear to remain stable across season and with T manipulations (O'Bryant and Wade, 1999). In contrast, T enhances copulatory muscle, but not motoneuron, morphology during the BS (Holmes and Wade, 2004b). These separate studies have suggested differences in responses to seasonal and hormonal manipulations, but drawing firm conclusions about relative degrees of plasticity is difficult because the multiple levels of the nervous system have not been concurrently examined in the same animals (Holmes and Wade, 2004b; O'Bryant and Wade, 1999). Importantly, males in these experiments had no access to females, so potential effects of use and/or social stimuli on reproductive morphology were not addressed. The present experiment was therefore designed to investigate morphological differences in forebrain regions and the courtship and copulatory neuromuscular systems of male green anoles across hormonal and environmental conditions, which included both abiotic and social cues. Males were exposed to T or vehicle control under either BS or NBS temperatures and photoperiods, as well as to one of three levels of contact with females (Holmes and Wade, 2004b; O'Bryant and Wade, 1999)

Materials and Methods

Animals and Housing

Experimental adult male and stimulus female green anoles, captured from the field, were purchased during the summer BS and fall NBS from Charles Sullivan, Co. (Nashville, TN). Individuals of the two sexes arrived simultaneously in the lab. Females were group housed in 29 gallon aquaria, and males were individually housed in 10 gallon aquaria. Each aquarium contained sphagnum peat moss bedding, wooden dowels for perching, a water dish and basking rocks. Fluorescent, full-spectrum and incandescent lights simulated natural environmental conditions. BS animals were exposed to long days (14 hours of light) with room temperatures ranging from 28°C during the day to 19°C at night. NBS animals were exposed to short days (10 hours of light) and temperatures varying from 24°C during the day to 15°C at night. Incandescent spotlights placed on top of each cage provided animals with basking temperatures up to 10°C warmer than ambient. Humidity was consistently set at 70%, and aquaria were sprayed daily with water. Animals were fed crickets or mealworms three times a week during the BS and twice each week during the NBS. Procedures were performed in accordance with Michigan State University Institutional Animal Care and Use Committee and NIH guidelines.

Endocrine Manipulations

Following Isoflurane anesthesia, males from the BS and NBS were bilaterally gonadectomized while on ice and subcutaneously implanted with a Silastic capsule (7mm long x 0.7 mm ID x1.65mm OD) containing 5mm of packed testosterone propionate (T) or left blank (Bl). Each male was weighed and measured snout-to-vent (nose tip to cloaca; SVL). Stimulus females were ovariectomized and implanted with 5mm capsules

made from a slurry of sealant and 2mg estradiol benzoate that had been extruded through the tip of a 10cc syringe (without a needle).

Behavior Testing

Two weeks following surgery, males began a series of 15 minute behavior tests administered once on each of 14 consecutive days. Males were randomly assigned to one of three female exposure conditions: (A) a different stimulus female was placed in each male's cage each day; (B) a glass aquarium containing a stimulus female was put immediately next to his; or (C) an empty cage was placed next to it. The number of dewlap extensions and copulations were recorded by an observer blind to hormone manipulation.

Tissue Collection

One day following behavior testing, males were overdosed with Sodium Pentobarbitol, and SVL and body weight were recorded a second time. Males were intracardially perfused with phosphate buffered saline followed by 10% phosphate buffered formalin. Evidence of the Silastic capsule's presence was confirmed at this time. Immediately following perfusion, the brain was removed and post-fixed in phosphate buffered formalin for seven days. Segments T15 through S2 of the spinal column, the ventral portion of the rostral tail containing the hemipenes and RPM muscles, the throat including ceratohyoid muscles, and the kidneys were collected in Bouin's fixative for 7 days. All tissues were soaked in 70% ethanol, dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin. Brains and spinal cords were

sectioned at 20μm. Tails, throats and kidneys were sectioned at 10μm. Tails and throats were stained with the trichrome method (Holmes and Wade, 2004b), brains and spinal cords were stained with thionin, and kidneys were stained with hematoxylin and eosin.

Morphological Measures

Using Scion (NIH) Image software, soma sizes of 20 randomly chosen neurons in the POA and AMY (ventromedial nucleus), 15 in AmbIX/VIImv, 10 in AmbX, and 20 in T17-S1 were measured on each side of each animal (Holmes and Wade, 2004b; O'Bryant and Wade, 1999; O'Bryant and Wade, 2002). Cross-sectional area was calculated from 25 ceratohyoid and 25 RPM fibers per side (Holmes and Wade, 2004b; O'Bryant and Wade, 1999). An average for each individual was calculated for each of these variables to be used in statistical analyses. Cross-sectional areas of TPN fibers were not determined because they run perpendicular to the RPM and thus cannot be measured in this preparation. An estimate of relative hemipene size was calculated by measuring 10 cross-sections, 50μm apart (Holmes and Wade, 2004b). Finally, the height of kidney epithelial cells in renal "sex segments" provides a bioassay for androgen exposure (Holmes and Wade, 2004b; Winkler and Wade, 1998), so the height of four such cells was measured in each of ten tubules randomly selected from the two kidneys. A mean from these 40 measurements in each individual was used for statistical comparisons.

Statistical Analysis

The sample sizes for all groups was 8, except for males treated with Bl capsules in the BS who were exposed to empty cages; one individual died during behavior testing

(n=7). The total number of dewlap extensions was analyzed with a 3-way ANOVA (hormone x season x female exposure condition). A 2-way ANOVA (hormone x season) was used for copulatory behavior because only males with a female present in the cage could copulate.

Average soma size for the POA, AMY, AmbIX/VIImv, AmbX and T17-S1, as well as hemipene, ceratohyoid and RPM muscle fiber size, kidney epithelial cell height, SVL and body weight were individually analyzed with 3-way ANOVAs (hormone x season x female exposure condition). For SVL and body weight, means of measurements recorded at the time of treatment and those taken just before males were perfused were used. Additional ANOVAs were performed to break down interactions when they existed, and Tukey-Kramer post-hoc tests were used for pairwise comparisons. Finally, some regression analyses and analyses of covariance were conducted as appropriate (details in Results).

Results

Behavior

Season, T and female exposure all had significant main effects on dewlap extension (Figure 1A). They were increased during the BS ($F=21.20$, $p<.0001$), with T treatment ($F=49.30$, $p<.0001$), and by at least visual exposure to females ($F=48.46$, $p<.0001$; Tukey-Kramer, control versus visual or physical contact, both $p<.05$). Significant interactions also existed, such that the effects of T ($F=5.82$, $p=.0180$) and female exposure ($F=3.90$, $p=.0240$) were greater during the BS than NBS. The effects of T were

also greater in males presented with females than control males (treatment x female exposure condition interaction: $F=10.12$, $p<.0001$).

Only males with physical access to females had the opportunity to copulate. For these animals, main effects of season and treatment existed (Figure 1B) such that only T-treated males ($F=49.47$, $p<.0001$) copulated, and they did so more frequently during the BS ($F=25.94$, $p<.0001$). T treatment had a greater effect during the BS than NBS ($F=25.94$, $p<.0001$).

Morphology

General Measures

Although males were randomly selected from shipments, they were on average 16% heavier ($F=20.70$, $p<.0001$; Table 1) and 3% longer in the NBS than BS ($F=8.07$, $p=.0057$; Table 1). No effects of T or female exposure existed on body weight or SVL. Renal sex segments were larger during the BS than NBS ($F=37.23$, $p<.0001$; Table 2) and in T- compared to Bl-treated males ($F=1352.09$, $p<.0001$). An interaction also existed, such that the effect of T was greater in the BS than NBS ($F=32.73$, $p<.0001$; Table 2).

POA and AMY

POA soma size was larger in T-treated animals than controls ($F=6.27$, $p=.014$) and under NBS compared to BS conditions ($F=6.34$, $p=.014$; Figures 2A and 3). T also increased AMY soma size ($F=5.59$, $p=.021$), and a season x T interaction existed in this region ($F=4.98$, $p=.029$) such that the effect of T was greater in the BS than NBS (Figures 2B and 3). No significant effects of female exposure existed on POA ($F=0.03$, $p=.974$) or

AMY (F=1.04, p=.360) soma sizes. Like POA soma size, body weight and SVL were greater in the NBS than BS (see above). Therefore, regression analyses were performed to determine whether a relationship between body size and POA soma size existed. Across all individuals, both body weight (R^2=.09, p=.005) and SVL (R^2=.08, p=.009) were positively correlated with POA soma size (data not shown). When potential effects of body weight and SVL on POA soma size were factored out by ANCOVA, the effects of season on POA soma size were eliminated (both F<2.96, p>.089).

Courtship Neuromuscular System

No significant effects of T or female exposure existed on ceratohyoid muscle fiber cross-sectional area (all F≤3.53, p≥.063). However, ceratohyoid muscle fiber size (F=7.31, p=.0083), and Amb IX/VIImv (F=21.59, p<.0001) and Amb X (F=24.83, p<.0001) motoneuron soma sizes were larger in the NBS than BS. As for the POA, these variables were each positively correlated with body weight (ceratohyoid: R^2=.17, p<.0001; Amb IX/VIImv: R^2=.06, p=.013; Amb X: R^2=.08, p=.0075) and SVL (ceratohyoid muscle: R^2=.11, p=.0013; Amb IX/VIImv: R^2=.06, p=.019; Amb X: R^2=.05, p=.040). The effects of season on the muscle and both brainstem motonuclei were eliminated if either body weight or SVL were factored out by ANCOVA (all F≤1.69, and p≥.196).

Copulatory Neuromuscular System

T increased the cross-sectional areas of hemipenis (F=116.23, p<.0001; Figure 4A) and RPM muscle fiber (F=46.01, p<.0001; Figures 4B and 5). The effects of T were

greater during the BS than NBS on both the hemipenis (interaction: $F=12.34$, $p=.0007$) and RPM ($F=10.18$, $p=.0020$; Figure 4). No main effects of season or female exposure were detected for the hemipenis or RPM (all $F \leq 1.51$, $p \geq .227$). No significant main effects or interactions existed for T17-S1 motoneuron soma size (all $F \leq 3.05$, $p \geq .085$; data not shown).

Discussion

Summary and comparison to previous anole work

The present results demonstrate that male sexual behavior in the green anole is facilitated by each of the three factors investigated: T, environmental conditions typical of the BS, and exposure to females. Interactions among these variables also indicate that the display of male sexual behaviors is more responsive to the effects of T and the presence of females during the BS than NBS, and to females when T levels are relatively high. Perhaps the most interesting results, however, involve the varied influences of hormone and seasonal conditions on the morphology of the different tissues. The peripheral copulatory system (hemipenes and RPM muscle fibers) substantially increased in size with T-treatment, whereas in the same individuals fibers of the muscle responsible for dewlap extension did not. T also appeared to induce growth in POA and AMY soma size, but not in the motonuclei responsible for dewlap extension or hemipene movement.

Importantly, parallel to reproductive behaviors, morphology of each of these structures, with the exception of the POA, was more responsive to T in the BS than NBS. The renal sex segments also showed a greater increase in cell size due to T in the BS compared to the NBS. These data suggest that this pattern is not limited to neural and

muscular structures, and that reduced responsiveness to T in the NBS may occur throughout androgen-sensitive tissues. While possible, it seems quite unlikely that the greater effects of T during the BS were due to a methodological difficulty, such as less T being released from the capsules in the NBS. Soma size in the POA was increased equivalently by T in the two seasons, which is consistent with the idea of comparable exposure. In addition, while it was not directly quantified, visual inspection suggested that approximately the same amounts of T remained in the capsules of BS and NBS males at the time of perfusion. Finally, while we did not collect plasma to measure T directly, other studies have found equivalent circulating T in the BS and NBS after T capsule implantation (Smith, et al., 1997b).

The behavioral and morphological effects of T and season that were detected in this study are consistent with and extend prior work, allowing for more complete conclusions. That is, as in the present study, others have documented that T is important for the display of male sexual behavior in the green anole (Lovern et al., 2004b; Wade, 2005). The present data also replicate T-induced increases in the size of hemipenes, copulatory muscle fibers and renal sex segment cell size, with greater effects under conditions typical of the BS than NBS (Holmes and Wade, 2004b; Holmes and Wade, 2005b; Lovern et al., 2004b; O'Bryant and Wade, 1999; Wade, 2005). Fewer statistically significant results were detected in the earlier study on the copulatory system, but the magnitude of the differences was nearly identical to those in the present study. Here, T induced approximately a 124% increase in hemipenis and RPM size in the BS and a 33% increase in the NBS. In Holmes and Wade (Holmes and Wade, 2004b), these values were 129% and 33% respectively. Thus, it is likely that the approximately 3-fold

increase in sample size contributed to interactions between season and T detected in the current, but not earlier, study. The lack of specific effects of T and seasonal environmental conditions in castrated males on the copulatory motoneurons and dewlap muscle fibers and motoneurons also replicate previous work (Holmes and Wade, 2004b; O'Bryant and Wade, 1999). Finally, an earlier study from our lab indicated that the soma size of neurons in the POA and AMY is not increased by T when administered under conditions typical of the NBS. However, it did not investigate the effect of the hormone on these cells in the BS. The present data are consistent with those results, particularly for the AMY in which a season x T-treatment interaction existed. They also indicate that if BS animals are added to the analysis, T does facilitate an increase in the size of cells in these regions. Thus, by evaluating behavior as well as morphology in three portions of the nervous system (limbic forebrain, and dewlap and copulatory neuromuscular) in the same individuals, we have been able to document clear and consistent differences in behavioral and morphological responsiveness to T at multiple levels – across structures, as well as between seasons.

The present data also for the first time consider the specific effects of female exposure. While it is no surprise that at least visual exposure to females is required to stimulate male sexual behavior, the fact that it had no direct effect on the morphology of any structure that was evaluated is quite intriguing. These results suggest that there are limits on the environmental cues that stimulate morphological change (that abiotic rather than social stimuli are relevant), and perhaps more importantly, the data are consistent with the idea that increases in use do not cause changes in the sizes of these neural or muscular cells. It is possible that a greater range in the total number of dewlap

extensions or copulations would be required to detect such an effect, but if it exists, it is likely that it is quite subtle.

The question, of course, is why the behavioral and morphological differences in responsiveness to T exist. On a functional level, morphology of structures important for dewlap extension may need to be maintained because the dewlap is used during the NBS (although far less frequently than the BS), in contrast to copulation which occurs rarely if ever in the NBS (Jenssen, et al., 1996; Lovern et al., 2004b; O'Bryant and Wade, 2002). On the other hand, T's increase of hemipenis size during the BS might serve a number of purposes, including facilitation of sperm delivery. And, a larger hemipene might require larger RPM muscle fibers. Increases in soma size in the POA and AMY in response to T may have less to do with "mechanics" and instead reflect the changes in function associated with integration of environmental cues and the hormonal status of a male to determine whether mating is appropriate (Swann, et al., 2003).

T-induced changes in morphology and behavior may involve androgen receptor (AR) expression. ARs are expressed in the anole POA and AMY during the BS (Rosen et al., 2002), but further work is needed to determine whether AR expression is modified by either T or environmental conditions. We do have information about neuromuscular structures, however. ARs are located throughout the copulatory system, and exogenous T increases their expression in the hemipenes and RPM, although it does not increase the percent of AR-positive T17-S1 motoneurons (Holmes and Wade, 2005b). ARs are also expressed in ceratohyoid muscles and 45% of AmbX motoneurons, but apparently not in AmbIX/VIImv motoneurons in intact males (Holmes and Wade, 2005b; Rosen et al., 2002). Unlike the copulatory system, but consistent with the relative lack of plasticity, T

does not seem to alter AR expression in dewlap structures (Holmes and Wade, 2005b). Therefore, T may enhance morphology of peripheral copulatory, but not courtship, structures at least in part via an up-regulation of AR.

The hormone may also influence morphology through other mechanisms. For example, aromatase activity is greater in whole brain homogenates from breeding compared to non-breeding adult male green anoles (Rosen and Wade, 2001). Aromatase activity (estradiol) is not required for the display of masculine behavior in this species (Winkler and Wade, 1998); T itself is the most potent activator of male sexual behavior (Wade, 2005). However, the effect of estrogen on brain morphology has not been investigated in this species. It is therefore possible that T's increase of POA and AMY soma size is mediated by estrogen receptors and/or an increase in aromatase activity, although at this point we do not know whether neural aromatase is increases by T in green anoles. Likewise, potential roles of other steroid-related factors that may change seasonally, such as steroid binding globulins and receptor co-activators have not yet been explored.

Reproductive Behavior and Morphology in Other Species

Like the anole, behavioral and morphological plasticity exist in other vertebrate species. For example, some seasonally breeding male songbirds sing to court females and copulate with them during the BS when T levels are high, and these behaviors diminish during the NBS when T levels decline (Ball and Balthazart, 2004; Prendergast et al., 2002). Parallel to behavioral changes, the volume of brain regions controlling song can increase due to the rise in T that occurs in the BS (Ball and Balthazart, 2004; Ball et

al., 2002; Brenowitz, 1997; Caro et al., 2005; Nottebohm et al., 1986; Rasika et al., 1994; Thompson and Brenowitz, 2005; Tramontin and Brenowitz, 2000; Tramontin, et al., 2003). However, unlike the anole, increased singing behavior in some birds contributes directly to enhancement of the morphology of song control nuclei (Alvarez-Borda and Nottebohm, 2002; Brenowitz and Beecher, 2005; Tramontin et al., 1999).

T and/or breeding environmental conditions enhance male sexual behavior and in parallel enhance volume and/or soma size of the POA and AMY in Japanese quail, whiptail lizards and rodents (Cooke, 2005; Cooke et al., 2003; Cooke et al., 1999; Crews et al., 1993; Panzica et al., 1996; Romeo and Sisk, 2001; Thompson and Adkins-Regan, 1994; Wade, 2005; Wade and Crews, 1991; Wade et al., 1993). Interestingly, as in the present study, a seasonal environmental cue (photoperiod) influences the response of a portion of the AMY (the MeApd) to T in Siberian hamsters. Soma size in and volume of this brain region are increased by treatment with this hormone to a greater extent in males housed in long- than short-days. These data parallel those indicating that photoperiod mediates the ability of T to regulate gonadotropin secretion, neuropeptide immunostaining, opiate binding and androgen receptor expression in particular brain regions in these animals, as well as those documenting that androgen fails to restore mating behavior in Syrian hamsters housed on short days (Bittman, et al., 2003; Cooke, 2006).

Exogenous T, as well as naturally occurring seasonal increases in T, also induce masculine reproductive behaviors and enhance muscle and/or motoneuron size in courtship structures in some birds, frogs, fish and copulatory muscle and/or motoneuron size in mammals (Arnold and Breedlove, 1985; Bass and Marchaterre, 1989; Breedlove

and Arnold, 1980; Breedlove and Arnold, 1981; Breedlove and Arnold, 1983; Cooke et al., 1998; Forger and Breedlove, 1987; Forger et al., 1992; Forger et al., 1996; Gurney, 1981; Gurney, 1982; Hegstrom et al., 2002; Kay et al., 1999; Kelley, 2004; Kelley et al., 1988; Park et al., 2002; Potter et al., 2005; Rand and Breedlove, 1995; Wade and Buhlman, 2000; Wetzel and Kelley, 1983). However, morphology and behavior do not respond to T and seasonal environmental conditions in parallel in all cases. For example, in free-living male song sparrows the volume and soma size of song control regions differ between the BS and NBS but song repertoire size does not (Smith, et al., 1997a). In wild canaries, seasonal rises in T and learning of new song syllables do not result in morphological changes to forebrain song production areas (Leitner, et al., 2001), but in a captive population of canaries song production areas show seasonal increases in volume parallel to increases in song learning (Nottebohm, 1981). In tree lizards, seasonal fluctuations in the volumes of the POA and AMY exist, but hormones appear to play a limited, if any, role. And, levels of aggressive behavior (measured rather than reproduction) are not associated with these differences (Kabelik, et al., 2006). Other examples of differential effects on morphology and function include facilitation of masculine sexual behaviors by T in whiptail lizards of both sexes, but T increases POA volume only in males (Wade et al., 1993). Similarly, larger mean muscle mass and motoneuron soma size are associated with *decreased* copulatory behavior in male rats (Breedlove, 1997; Raouf et al., 2000).

Conclusions

The POA and AMY, plus the dewlap and copulatory neuromuscular structures are components of a circuit necessary for the display of the full suite of reproductive behaviors in the male green anole. However, differences in the degree of plasticity exist across these levels; they respond uniquely to seasonal and hormonal cues. Investigating multiple levels of this reproductive system has provided an integrated picture of relationships among T, morphology and behavioral function in green anoles, including seasonal and structural differences in the sensitivity to T. In some cases, these effects parallel those in other vertebrate groups. However, critical differences exist, perhaps including a relatively unique change in the structural and functional responses of tissues to T across seasons. To our knowledge, the issue of differences in responsiveness to T across seasons, particularly the variation among tissues responsible for coordinating different levels of reproductive behaviors, has not been considered fully in other model systems. It will now be important to investigate in depth the mechanisms behind the potentially differential sensitivity to T, both across structures and seasons, in particular because the most obvious mediator (level of AR expression) does not appear directly responsible.

Acknowledgments

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Table 1. Mean ± SEM Body Weight and Snout-to-Vent Length (SVL) During the Breeding (BS) and Non-breeding (NBS) Seasons.

	BS*	NBS
Body Weight (g)	4.40 ± 0.07	5.09 ± 0.12
SVL (mm)	61.08 ± 0.33	63.00 ± 0.56

* significant effect of season. Values represent averages across hormone treatment and female exposure conditions because these variables had no effect on body weight or SVL.

Table 2. Mean ± SEM Renal Sex Segment Cell Height in Testosterone (T) and Blank (Bl) Treated Males During the Breeding (BS) and Non-breeding (NBS) Seasons.

	BS*	NBS
T#	46.60 ± 1.12	37.20 ± 0.99
Bl	12.87 ± 0.29	12.52 ± 0.26

* significant effect of season; # significant effect of T; an interaction between these manipulations was also detected. Measurements are averaged across female exposure conditions because this variable did not affect cell height.

Figure 1. Total number of dewlap extensions (A) and copulations (B) under environmental conditions typical of the breeding and non-breeding seasons in gonadectomized males implanted with either testosterone (T) or blank (Bl) capsules, and exposed to one of three levels of female contact. Statistically significant main effects of season, hormone treatment and female exposure condition, as well as interactions among season and hormone treatment, season and female exposure, and hormone treatment and female exposure were detected for total dewlap extensions. Main effects of season and hormone treatment and an interaction between season and hormone treatment existed in males with female contact for total copulations.

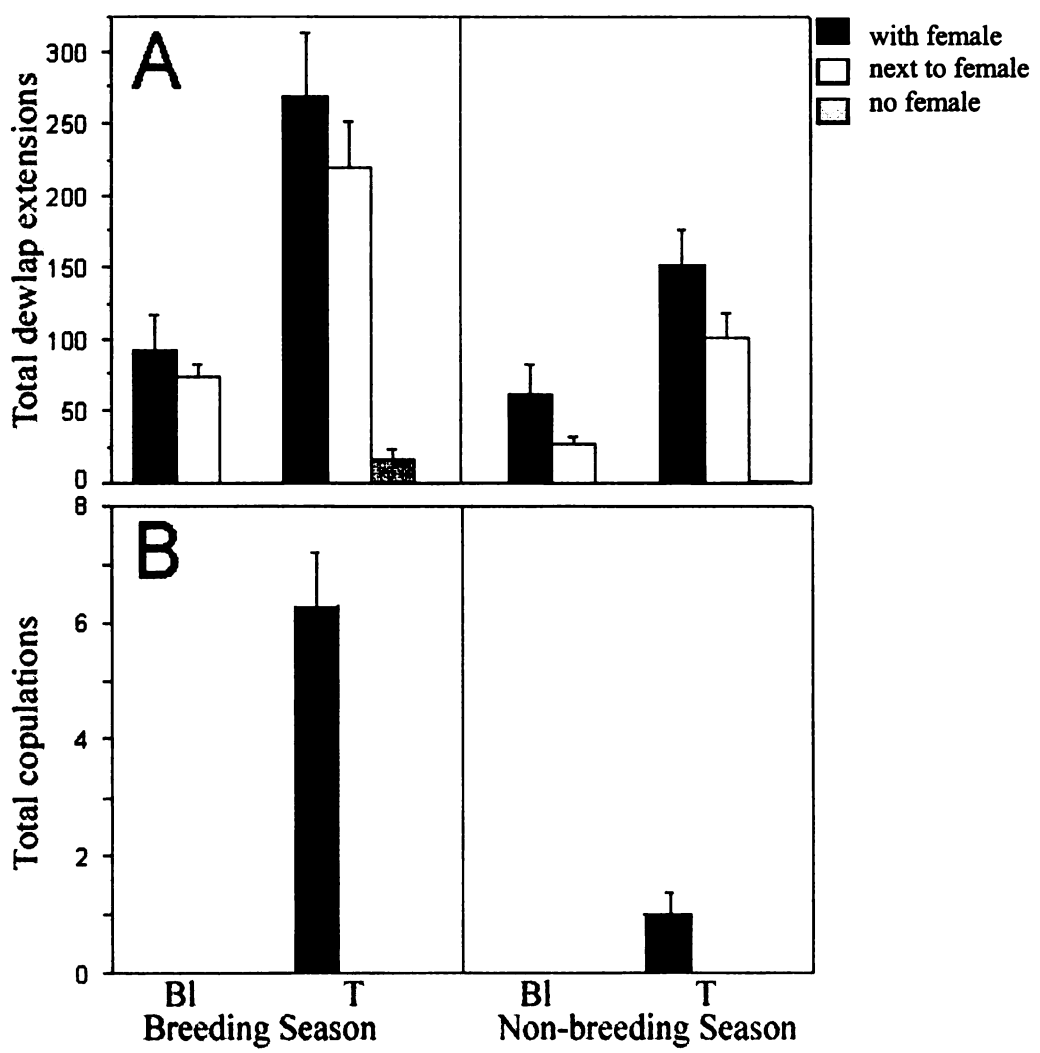


Figure 2. Soma size in the preoptic area (POA) (A) and amygdala (AMY) (B) under environmental conditions typical of the breeding and non-breeding seasons in gonadectomized males implanted with either testosterone (T) or blank (Bl) capsules, and exposed to one of three levels of female contact. Means ± standard errors are depicted.

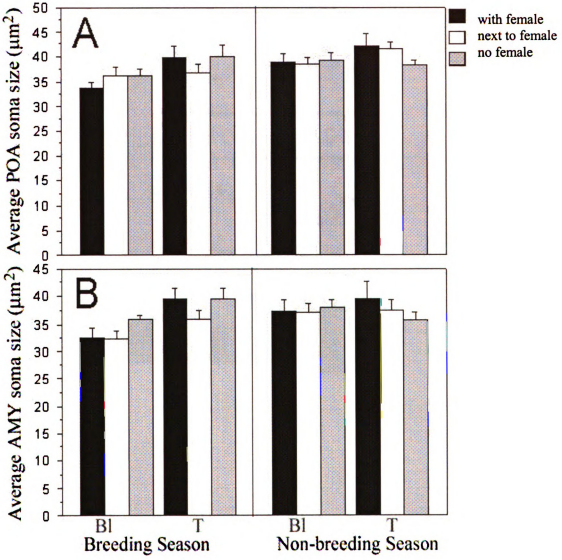


Figure 3. Photomicrographs of neurons in the preoptic area (A and B) and amygdala (C and D) taken from breeding condition males. (A) and (C) are from males treated with T , and (B) and (D) are from males implanted with Bl capsules. Scale bar = 10μm.

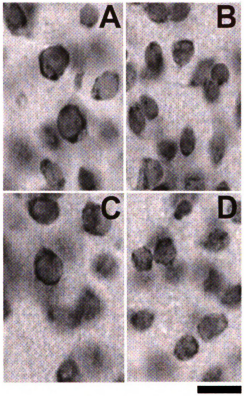


Figure 4. Cross-sectional area of the hemipenis (A) and retractor penis magnus (RPM) fibers (B) under environmental conditions typical of the breeding and non-breeding seasons in gonadectomized males implanted with either testosterone (T) or blank (Bl) capsules, and exposed to one of three levels of female contact. Mean values ± standard errors are depicted for each group. Main effects of season and an interaction between season and hormone treatment existed for both hemipenis (A) and RPM fiber size (B).

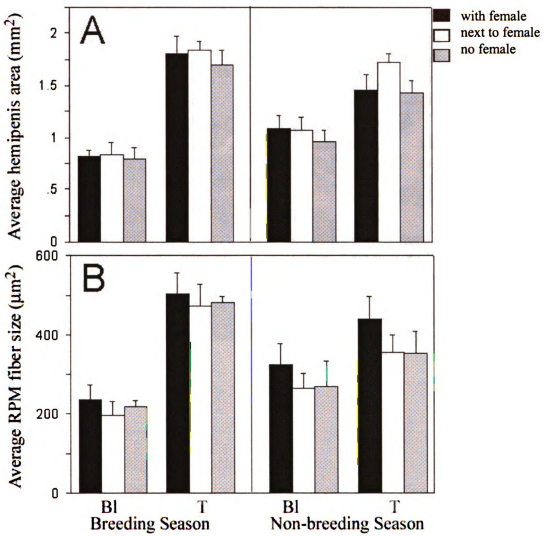
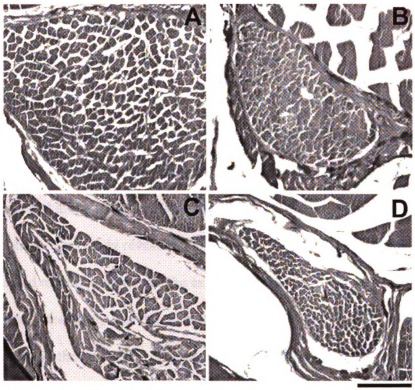


Figure 5. Photomicrographs of cross-sections through RPM muscle fibers under environmental conditions typical of the breeding (A and B) and non-breeding (C and D) seasons. (A) and (C) are from T-treated, and (B) and (D) from Bl-treated males. Scale bar = 100μm.



CHAPTER 3: ANDROGEN RECEPTOR EXPRESSION AND MORPHOLOGY OF FOREBRAIN AND NEUROMUSCULAR SYSTEMS IN MALE GREEN ANOLES DISPLAYING INDIVIDUAL DIFFERENCES IN SEXUAL BEHAVIOR

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 confers this difference in responsiveness.

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., 2004b; 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/VIImv) 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, 2005b), the ceratohyoid muscles, and about 45% of Amb X neurons, but have not been detected in AmbIX/VIImv (Holmes and Wade, 2005b; 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, 2005b; 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 lab 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 hours 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 5mm long capsules created from a slurry of Silastic sealant (Dow Corning; Midland,

MI) and 2mg estradiol benzoate (Steraloids; Wilton, NH) that had been extracted through the tip of a 10cc syringe. A novel female was introduced into the cage of each of 44 males for 15 minutes per day for 7 consecutive days. All behavior tests were run between 1000-1300 hours (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 minute 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 minute test in which they might have courted, or if the male did not copulate, the entire 15 minute 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; Figure 6A). Studs extended their dewlaps at a significantly greater rate than duds ($t=11.09$; $p<0.001$; Figure 6B). 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 $t<.98$; all $p>.338$; data not shown).

Tissue Collection

Approximately 24 hours 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 20μm in 6 series, and stored at –80°C until processing for AR immunohistochemistry (as in Holmes and Wade, 2005b) 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; Lovern and Wade, 2001). Briefly, the samples were thawed, mixed with 0.5 mL dH_2O and equilibrated overnight at 4°C with 1000 cpm of 3H-T (NET-370, 95 Ci/mmol; 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 1,000 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 centifuged, 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/VIImv, 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 genioglosus 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 {total of 40 measurements; \Holmes, 2004 #2;Neal, 2007 #525;Winkler, 1998 #52}. 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 (2005b) 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 minutes. After rinsing in 0.1 M PBS, tissue was incubated in 0.5% hydrogen peroxide for 30 minutes followed by 2 hours 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 hours in PG-21 rabbit polyclonal antibody (1.75ug/ml for cords, throats and tails; 3.5ug/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 minute 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 x 100μm^2 (POA) or 100 x 100μm^2 (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 225μm dorsal to the optic chiasm just above the suprachiasmatic nucleus and the long edge of the box was about 40μm lateral to the third ventricle. In the AMY, the boxes were placed 50μm 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 x 200μm^2 (RPM) or 500 x 400μm^2 (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, 2005b). 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 lab (Holmes and Wade, 2005b).

As in Holmes and Wade (2005b), 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; Holmes and Wade, 2005b; 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/VIImv do 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 0.18-8.21 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, 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: $r=0.40$; $p=0.037$; Figure 7A; copulations:$r=0.41$; $p=0.032$). Plasma androgen concentration did not differ between studs and duds ($t=1.88$; $p=0.081$; Figure 7B).

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 ($r=0.49$; $p=0.008$; Figure 7C) and total number of copulations ($r=0.46$; $p=0.015$; not shown), and it was greater in studs than duds ($t=2.40$; $p=0.032$; Figure D).

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 ($r=0.39$; $p=0.047$; Figure 8), 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 ($r=0.44$; $p=0.017$; Figure 9A) and was significantly larger in studs than duds ($t=4.03$; $p=0.001$; Figure 9B and 10). 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/VIImv and AmbX soma sizes were not correlated with expression of either behavior (all $r \leq 0.14$; all $p \geq 0.414$). No statistically significant differences existed between studs and duds in AmbIX/VIImv ($t=0.36$; $p=0.725$) 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; Holmes and Wade, 2005b; 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, 2005b), up-regulation

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 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 genioglosus, 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., 2004b; 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 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 one 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 recrudesce 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., 2004b), 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 enhance 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; Morgantaler and Crews, 1978; Noble and Greenberg, 1941; Wade et al., 1993; Wheeler and Crews, 1978; 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 immunohistochemical 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.

Acknowledgements

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Figure 6. 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 minute 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 $\pm$ standard errors; * = $p<0.0001$).

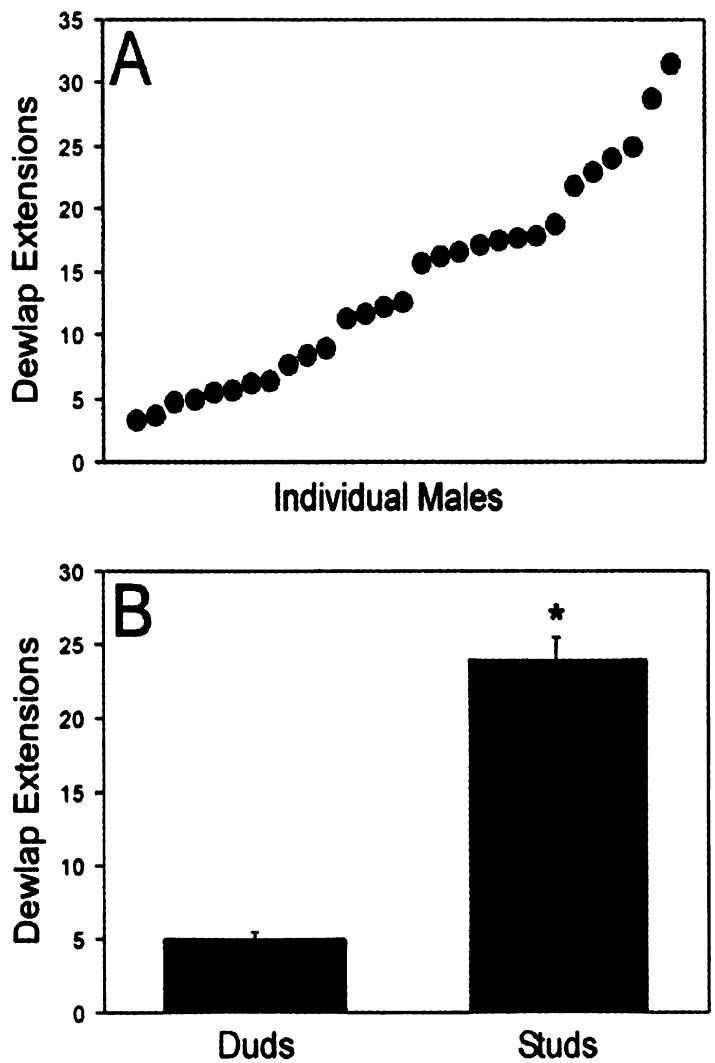


Figure 7. Relationships between behavior and plasma androgen (A and B) or renal sex segment height (C and D). Panels A and C depict correlations of the two physiological measures with dewlap extension rate. Panels B and D indicate comparisons between high- and low-intensity courters. The open circle in A indicates the outlier (means ± standard errors; N.S. = not statistically significant).

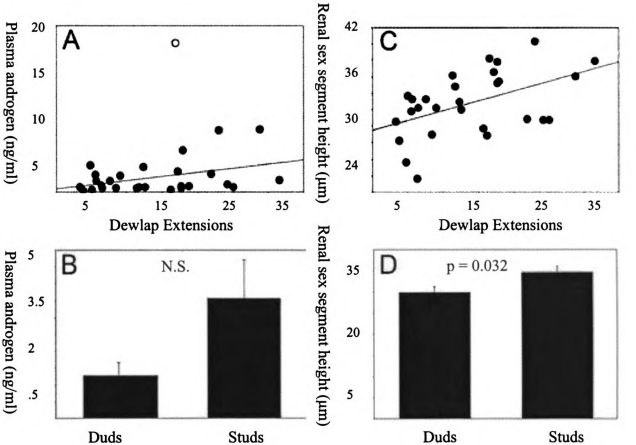


Figure 8. Average amygdala (AMY) soma size was positively correlated with the rate of dewlap extensions.

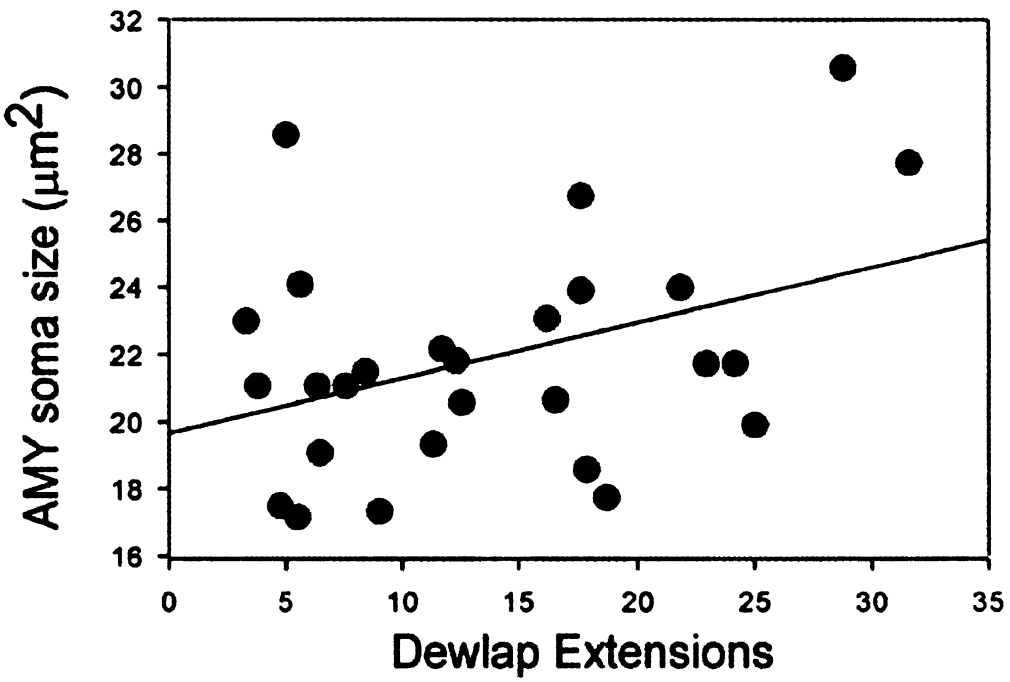


Figure 9. Relationships between muscle fiber size and dewlap extension rate. Panel A depicts a significant correlation (p=0.017), and panel B documents the mean group difference between studs and duds (means ± standard errors; p=0.001).

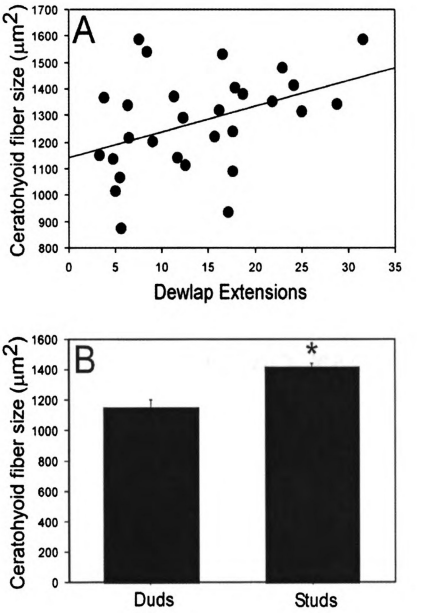
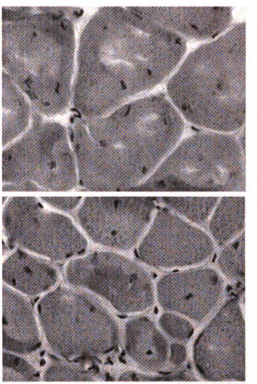


Figure 10. Photomicrographs of cross-sections through ceratohyoid muscle fibers in a stud (top) and dud (bottom). Scale bar = 50μm.



CHAPTER 4: EFFECTS OF SEASON, TESTOSTERONE AND FEMALE EXPOSURE ON C-FOS EXPRESSION IN THE PREOPTIC AREA AND AMYGDALA OF MALE GREEN ANOLES

Abstract

Expression of the immediate early gene, c-fos, was used to investigate neuronal activity in forebrain regions involved in male sexual behavior following social, hormonal and/or seasonal manipulations in the male green anole lizard. These factors all influence behavior, yet it is unclear how they interact to affect neuronal activity in the forebrain, including in the preoptic area (POA) and ventromedial nucleus of the amygdala (AMY). These regions are involved in the display of sexual behaviors in male green anoles as in many other vertebrates. To determine the effects of seasonal, hormonal and social cues on these brain areas, we investigated c-fos under environmental conditions typical of the breeding or non-breeding season in adult male green anoles that were castrated and implanted with either testosterone (T) or blank (Bl) capsules. We also manipulated social cues by exposing only half of the animals in each group to females. T enhanced courtship and copulatory behaviors, but decreased c-fos expression in the AMY. A similar, although not statistically significant, pattern was observed in the POA, and the density of c-fos+ cells was negatively correlated in that region with the number of extensions of a throat fan (dewlap) used during courtship. Therefore, it appears that in the male green anole, T may diminish c-fos expression in the anole forebrain (likely in inhibitory neurons) to create a permissive environment in which the appropriate behavioral response can be displayed.

Introduction

Limbic forebrain regions, including the preoptic area (POA) and amygdala (AMY), are involved in the expression of male sexual behaviors. Neuronal activity in these regions, indicated by expression of immediate early genes such as c-fos, is associated with the display of male behaviors in a diverse array of vertebrates (Greco et al., 1996; Greco et al., 1998; Heeb and Yahr, 1996; Kollack-Walker and Newman, 1995; Kollack-Walker and Newman, 1997; Mello, 2002; Shimura et al., 1994). These areas also express steroid receptors in numerous vertebrate species (Ball et al., 2004; Hull et al., 2002; Wade, 2005), and testosterone (T), and/or its metabolites, estradiol and dihydrotestosterone, activate courtship and copulatory behaviors in males of many species, often on a seasonal basis (Ball et al., 2004; Ball and Balthazart, 2004; Cooke, 2006; Cooke et al., 1999; Lovern et al., 2004b; Prendergast et al., 2002; Romeo et al., 2001; Sakata et al., 2003; Wood and Swann, 2000). Environmental conditions can interact with hormone levels to affect the expression of immediate early genes. For example, following exposure to females, c-fos expression in the POM (the avian homologue of a portion of the mammalian POA) of male starlings, is positively correlated with behavior during the breeding season (BS; when T levels are high), but not non-breeding season (Heimovics and Riters, 2005; Heimovics and Riters, 2006). Social cues can also directly influence neuronal activity; c-fos is greater in reproductive forebrain nuclei in rodents and songbirds following social contact (Mello, 2002; Schwab et al., 2004). While many studies have examined the effects of seasonal, hormonal and social cues on c-fos expression separately, few have investigated potential interactions

among them, fewer have examined c-fos in reptilian brains (Bertolucci, et al., 2000) and none to our knowledge have done so in green anoles.

This species is ideal for conducting this type of work. The animals breed seasonally and exhibit naturally occurring behavioral plasticity. During the BS, males extend a bright red throat fan (dewlap) to court females, and copulation involves intromission of one of two bilateral hemipenes (Crews, 1980; Greenberg and Nobel, 1944; Jenssen et al., 2000). Lesion studies document that the AMY (its ventromedial nucleus) and POA facilitate male reproductive behaviors in lizards, as in other vertebrates (Bass and Zakon, 2005; Crews and Moore, 2005; Greenberg et al., 1984; Morgantaler and Crews, 1978; Murphy and Hoffman, 2001; Panzica et al., 1996; Wade, 2005; Wilczynski et al., 2005; Wood and Swann, 2000; Yahr and Gregory, 1993). Courtship and copulatory behaviors in green anoles are facilitated by environmental conditions typical of the BS, and are greatly diminished during the NBS. T, rather than its metabolites, is the primary activator of these behaviors in males (Adkins and Schlesinger, 1979; Crews et al., 1978; Greenberg and Nobel, 1944; Jenssen et al., 2000; Lovern et al., 2004b; Neal and Wade, 2007; O'Bryant and Wade, 1999; Rosen and Wade, 2000; Wade, 2005; Winkler and Wade, 1998). T and seasonal environmental conditions also affect forebrain morphology in the anole. Soma size of POA and AMY neurons is increased by T, and an interaction exists in the AMY, such that the hormone enhances soma size more in the BS than NBS (Neal and Wade, 2007; O'Bryant and Wade, 2002). Androgen receptors are present in the POA and AMY (Rosen et al., 2002), suggesting that T may act directly in these regions. While at least visual exposure to females is required for males to display courtship behaviors, neither visual nor direct female contact affects POA or AMY

morphology (Neal and Wade, 2007). However, in intact males, larger soma size in the AMY is associated with higher rates of dewlap extensions (Neal and Wade, submitted).

To determine whether T, season and/or female contact alter neuronal activity, expression of c-fos was examined in the POA and AMY in adult male green anoles. Animals were exposed to T or control vehicle under either BS or NBS temperatures and photoperiods, as well as to one of two levels of female contact (exposed to a female, or not).

Experimental Procedure

Animals and Housing

Wild-caught, adult male green anoles were purchased in the season in which they were tested (BS or NBS) from Charles Sullivan, Co. (Nashville, TN). Prior to use in the experiment, males were individually housed in 10 gallon glass aquaria, and adult females were group housed in 29 gallon aquaria for 1.5 weeks. Environmental conditions typical of the BS (14 hours of light; room temperatures ranging from 28°C during the day to 19°C at night) or NBS (10 hours of light; temperatures varying from 24°C during the day to 15°C at night) were maintained with fluorescent, full-spectrum and incandescent lights. Basking temperatures were up to 10°C warmer than ambient from incandescent spotlights placed on top of each cage. Aquaria were sprayed daily with water, and the humidity was consistently set at 70%. Sphagnum peat moss bedding, wooden dowels for perching, a water dish and basking rocks were provided in each aquarium. During the BS, animals were fed crickets or mealworms three times a week and during the NBS, twice each week. All procedures were performed in accordance with Michigan State University Institutional Animal Care and Use Committee and NIH guidelines.

Endocrine Manipulations

Males from the BS and NBS were anesthetized by hypothermia, gonadectomized, and subcutaneously implanted with a Silastic capsule (7mm x 0.7 mm ID x 1.65mm OD). Implants were either packed with testosterone proprionate (5mm of hormone), or left empty (blank; Bl). Capsules containing 10% estradiol benzoate (5mm long) were made from a slurry of Silastic sealant (Dow Corning; Midland, MI) and 2mg estradiol benzoate extruded through a 10cc syringe. They were subcutaneously implanted in ovariectomized females to facilitate receptivity.

Behavior Testing

Two weeks after receiving implants, males were randomly assigned to one of two female exposure conditions. They either received a stimulus female placed directly in their cage or no female. The number of dewlap extensions and copulations by each male was recorded for 1.5 hours by an observer blind to hormone manipulation.

Tissue Collection

Immediately following behavior testing, males were rapidly decapitated. Brains and kidneys were removed and frozen in isopentane, and stored at –80 °C. The Silastic capsule's presence and condition was confirmed at this time. Brain and kidney tissues were sectioned in six series on a cryostat at 20μm and stored at –80 °C. Brains were processed for c-fos immunohistochemistry and kidneys were stained with hematoxylin and eosin.

Immunohistochemistry

Each male was processed for c-fos immunohistochemistry (Bailey, et al., 2002; Bailey and Wade, 2003; Bailey and Wade, 2005). Briefly, after warming slides to room temperature, sections were rinsed, fixed in 4% paraformaldehyde and incubated in 0.5% hydrogen peroxide for 30 minutes followed by a one hour incubation in 4% normal donkey serum in 0.1 M PBS with 0.3% Triton X-100. Tissue was incubated for 72 hours at 4 °C in c-Fos primary antibody (1:1,000; Santa Cruz Biotech, Santa Cruz, CA, #sc-253) in 0.1 M PBS with 0.3% Triton X-100 and 30% glycerol. Following a series of rinses, tissue was incubated in biotinylated donkey anti-rabbit secondary antibody (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA). The Elite ABC kit solution (Vector; Burlingame, CA), and nickel-enhanced diaminobenzidine (Sigma; St. Louis, MO) were used to visualize c-fos immunoreactivity. To ensure that non-specific labeling had not occurred, the primary antibody was omitted, and no labeling was detected.

C-fos+ cells were assessed in the forebrain of every individual in a 250μm x 100μm (POA) or 100μm x 100μm (AMY) box placed in 3-5 sections (depended on quality of tissue) from each brain area on both sides. This procedure resulted in 6-10 values per individual. The long edge of the box was placed 40μm lateral to the third ventricle, and the short edge of the box approximately 225μm dorsal to the optic chiasm just above the suprachiasmatic nucleus in the POA (Figure 5). The AMY box was placed in the center of the medial-lateral extent of the nucleus and 50μm dorsal to the ventral edge of the

brain (Figure 3). The number of c-fos+ cells was averaged within each brain region in each animal for use in statistical analyses.

In ten tubules randomly selected from the two kidneys (total of 40 measurements), the height of four epithelial cells was measured for a total of 40 measurements. These values provide an indication of relative androgen exposure (see (Holmes and Wade, 2004b; Neal and Wade, 2007; Winkler and Wade, 1998).

Statistical Analysis

Sample sizes for all groups was 9, except that the tissue of one Bl-treated male exposed to a female during the NBS was damaged (n=8). The total number of dewlap extensions, copulations, average kidney epithelial cell height and density of c-fos+ cells in the POA and AMY were separately analyzed with a 3-way ANOVA (hormone x season x female exposure condition). Correlations were conducted to determine the degree to which the number of dewlap extensions and c-fos expression in the POA and AMY were associated. All statistical analyses were computed with StatView (SAS Institute, Inc; Cary, NC).

Results

Behavior

As expected (see Introduction), T increased dewlap extensions ($F = 13.34$, $p < 0.005$), as did BS conditions ($F = 7.82$, $p = 0.007$) and exposure to females ($F = 70.62$, $p < 0.001$). A significant interaction existed between hormone treatment and social contact such that the effects of T were greater in males presented with females than those who

were not ($F = 8.05$; $p = .006$; Figure 11, top). No other interactions were detected (all $F < 2.57$; $p > 0.114$). For copulatory behavior, main effects of treatment, season and female exposure, as well as all possible interactions among the variables, were statistically significant (all $F = 64.0$; $p < 0.001$); only T-treated males in the BS that were exposed to a female copulated (Figure 11, bottom).

AMY

T decreased the density of c-fos+ cells in the AMY ($F = 5.13$; $p = 0.027$; Figure 12, top and Figure 13). No main effects of season or female exposure or interactions between variables existed (all $F \leq 1.29$; all $p \geq 0.260$). Among males exposed to females, the density of c-fos+ cells in the AMY was not significantly correlated with the number of dewlap extensions ($r = 0.09$; $p = 0.617$; data not shown).

POA

The pattern of expression in the POA was generally similar to that of the AMY. However, the density of c-fos+ cells in the POA did not differ significantly between groups, and no interactions were detected (all $F \leq 2.42$; all $p \geq 0.125$; Figure 12, bottom). A negative correlation did exist in this region between the number of dewlap extensions and the density of c-fos+ cells in males exposed to females ($r = 0.45$; $p = 0.006$; Figure 14). When this correlation was broken down by group, it was detected only in T-treated males in the BS ($r = 0.74$; $p = 0.022$; Figure 14 and 15).

Renal sex segments

The height of epithelial cells in the kidneys (renal sex segments) was measured as an indication of androgen exposure. Main effects of T and season existed such that cell height was larger in T- than Bl-treated males ($F = 201.09$, $p < 0.001$) and during the BS than NBS ($F = 103.28$; $p < 0.001$). Also, a significant interaction was detected such that the effect of T was greater in the BS than NBS ($F = 100.75$; $p < 0.001$). These data (not shown) are consistent with those in other experiments (Holmes and Wade, 2004b; Holmes and Wade, 2005b; Neal and Wade, 2007; Winkler and Wade, 1998), and indicate that the hormone released from the capsule affected androgen-sensitive tissues as expected.

Discussion

Summary

In the present experiment, T activated male sexual behavior yet decreased the density of c-fos+ cells in the AMY. These results suggest that the hormone may have a disinhibitory effect, one which is independent of season and female presence, as these variables did not modulate expression of the immediate early gene. A similar pattern was revealed in the POA, although it was not statistically significant. Courtship behavior was, however, negatively correlated with the density of c-fos+ cells in the POA, which is consistent with the idea that either enhanced behavior decreases neuronal function or that activity of particular cells inhibits courtship displays. The latter idea fits well with the AMY data.

Potential functional explanations

The anole POA and AMY have a variety of characteristics associated with behavioral functions. For example, lesion studies in green anoles have determined that they are critical to the display of male courtship and copulation (Crews and Moore, 2005; Greenberg et al., 1984; Morgantaler and Crews, 1978; Wade, 2005). T enhances the reproductive behaviors as well as soma size in these areas, and its effects on courtship, copulation and AMY soma size are all greater in the BS than NBS (Neal and Wade, 2007; O'Bryant and Wade, 1999). As cells in the POA and AMY express AR (Rosen et al., 2002), T could act directly in these forebrain areas to facilitate both behavioral and morphological change, but we do not yet know whether seasonal differences in receptor expression exist in either region that might influence the differences in responsiveness during the BS and NBS.

The present data on immediate early gene expression support this idea that T can directly modify the activity of cells, at least in the AMY. And, with the exception of males in the BS that were not exposed to females, the pattern of T effects on c-fos in the POA and AMY were the same. In this group (BS males without females), however, T seemed to increase the density of c-fos+ cells in the POA, but it had the opposite effect in the AMY. As significant interactions between hormone manipulation and season or female exposure were not detected for either brain region, it seems unlikely that this one difference in the pattern of c-fos expression between the two brain regions is biologically meaningful. However, it did result in the main effect of T only reaching statistical significance for the AMY.

Still, a variety of differences have been detected between the two brain regions in green anoles. For example, in addition to the seasonal change in the effect of T on

morphology of the AMY but not POA (see above), the rate of dewlap extensions is positively correlated with soma size in intact adult males during the BS in the AMY, but not POA (Neal and Wade, submitted). In contrast, in the present study we documented that courtship display rate is negatively correlated with c-fos expression in the POA, and did not detect the same result for the AMY. Collectively, these differences between the two regions might reflect more specialized functions of these forebrain areas. The POA and AMY are reciprocally interconnected and both contribute to masculine courtship and copulation. However, at least in mammals, the POA is most likely the primary forebrain site for initiating the behaviors, whereas the AMY may be more involved in integrating sensory cues to affect motivation (Wood, 1997). While we can only speculate at this point, results from the green anole appear consistent with these functions. That is, it is possible that in this species T and environmental signals typical of the breeding season are integrated in the AMY, which results in both increases in soma size and behavioral output at the appropriate time. These two results may be causally related (behavior may increase soma size or vice versa) or they may be independent effects of the hormonal and external cues. Regardless, it is likely that one mechanism through which T acts in the AMY is to decrease c-fos expression, thus releasing some sort of tonic inhibition. The fact that this diminished neural activity is associated with the rate of dewlap extension in the POA but not AMY suggests that disinhibition in this region is more directly involved in the production of this behavior. However, the lack of a significant correlation between POA soma size and the rate of dewlap extension, combined with the fact that T enhances soma size in this area regardless of season, suggest that the display of courtship behavior displayed is not causally linked to neuron soma size in this area.

The idea that the function of inhibitory neurons is decreased is consistent with data from other species. For example, in male gerbils, c-fos and the inhibitory neurotransmitter GABA are co-localized in half of POA and AMY neurons, whereas only about one-fourth of the neurons co-express c-fos and the excitatory neurotransmitter glutamate, following mating (Simmons and Yahr, 2003). In addition, hormonal manipulations can modulate GABA receptor function (Clark and Henderson, 2003). For example, T-treatment in mice decreases the levels of individual GABAa receptor subunit mRNAs in the POA and AMY (McIntyre, et al., 2002). Studies in mice also suggest that activation of specific serotonergic receptors (such as 5HT1A, 5HT1B and 5HT2C) inhibit sexual motivation (Popova and Amstislavskaya, 2002a; Popova and Amstislavskaya, 2002b).

While immediate early gene expression has been widely used to identify brain regions involved in the control of sexual behavior, including those composing the "social behavior network" (Goodson, 2005; Newman, 1999), the results are not necessarily consistent. For example, c-fos expression is enhanced in the POA and AMY of male rodents, quail and house sparrows following exposure to a sexual stimulus and/or reproductive behavior (Heeb and Yahr, 1996; Pfaus and Heeb, 1997; Riters, et al., 2004; Taziaux, et al., 2006). In starlings, T and season interact to produce changes in immediate early gene expression associated with male sexual behavior; following contact with a female, c-fos expression in the POA is positively correlated with behavior during the BS when T levels are high, but not NBS when T is low (Heimovics and Riters, 2005; Heimovics and Riters, 2006). Also, c-fos expression is increased in rodents when males and females are housed together, thus experiencing social contact, compared to isolated

counterparts (Schwab et al., 2004). Immediate early gene expression is also increased in regions of the hypothalamus and suprachiasmatic nucleus of the female frog after hearing a socially relevant call compared to no sound or other signals (Hoke, et al., 2005). All of these results appear inconsistent with the idea that a decrease in the activity of inhibitory neurons permits the display of masculine sexual behaviors.

Yet, in other cases the data are similar to the present study. For example, immediate early gene expression in the frog POA does not differ between groups hearing socially relevant calls compared to those that do not (Hoke et al., 2005). In avian species, immediate early gene expression in the medial AMY is negatively correlated with group size of the species (Goodson, 2005; Goodson, et al., 2005a; Goodson, et al., 2005b). While it is difficult to draw a direct comparison between this research and the present study, the data are at least consistent with the idea that increased behavioral interactions can be associated with *decreased* rather than *increased* immediate early gene expression.

Cleary, relationships among internal physiological cues (such as hormones), external signals, neural activity and behavioral output are complex. Presumably, a balance between excitatory and inhibitory influences must be achieved, depending on the context. More direct comparisons across species evaluated under similar conditions should help to elucidate fundamental principles. In addition, more work to uncover underlying cellular mechanisms in diverse organisms is necessary.

Conclusions and Future directions

Collectively, the data from the present study are consistent with the idea that T may decrease activity of inhibitory neurons, making it more likely that if a female is

present, reproductive behaviors are facilitated in breeding males. Decreased inhibition in the AMY may increase motivation for masculine behaviors. Reduced inhibitory signals in the POA may produce (or be produced by) these behaviors. It will now be important to determine whether GABA and 5HT expression are influenced by T and/or female exposure in the male green anole. In particular, co-localization studies of these neurotransmitters with c-fos could reveal how they may influence the display of masculine sexual behavior. Similarly, it would be very useful to know whether neurons expressing androgen receptors are specifically active with the display of sexual behaviors, and whether in fact they are inhibitory. Associated molecular mechanisms (MacLean et al., 1997; O'Bryant and Jordan, 2005) likely also influence whether and how the activity of particular neurons modulate behavior. Finally, the entire complement of cells in these brain regions must be considered. That is, it is possible that different relationships would be detected by evaluating the expression of other immediate early genes (Bailey and Wade, 2003; Hoffman and Lyo, 2002; Hoke et al., 2005) or other biochemical markers of neuronal activity (oxidative metabolism, glucose uptake, etc.; e.g., (Tlemcani, et al., 2000). All of these factors should be investigated in future studies on the green anole.

Acknowledgments

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Figure 11. Number of dewlap extensions (top) and copulations (bottom) during the 1.5 hour behavior test. Males were castrated and implanted with either testosterone (T) or blank (Bl) capsules during the breeding and non-breeding seasons, and either exposed to females or not. For dewlap extensions, main effects of season, hormone treatment and female exposure condition existed, as well as an interaction between hormone treatment and female exposure (top). Main effects of season and T-treatment and an interaction between seasonal, hormonal and social manipulations were also detected for total copulations (bottom).

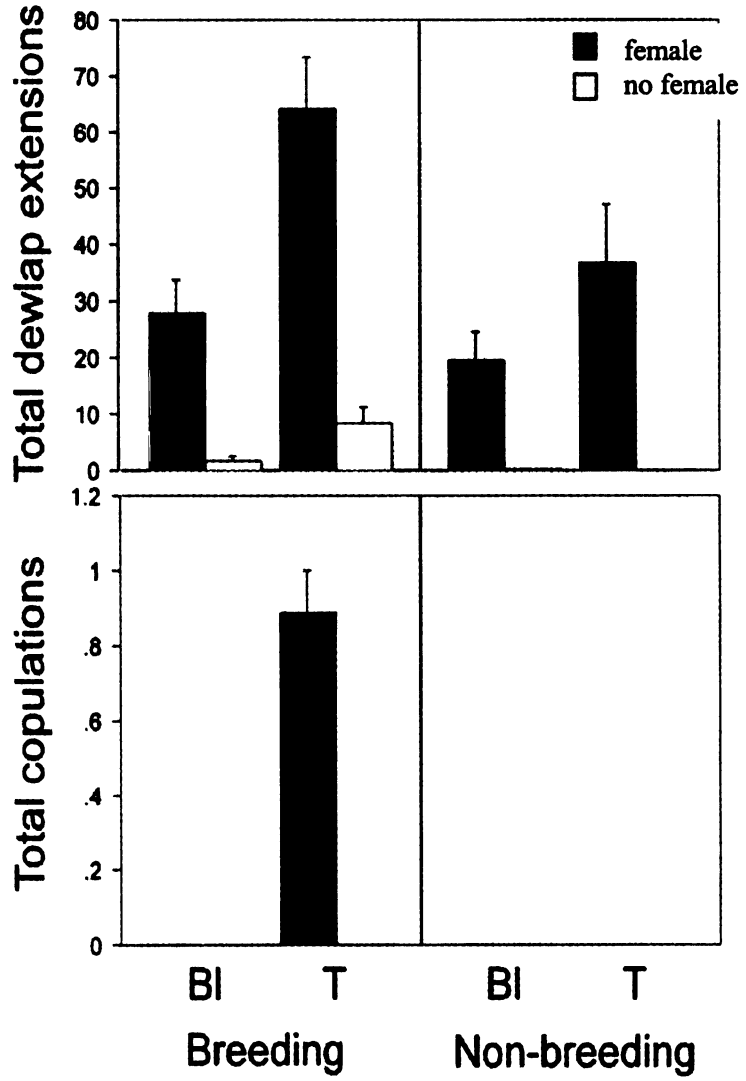


Figure 12. Average density of c-fos+ cells in the AMY (top) and the POA (bottom) under environmental conditions typical of the breeding and non-breeding seasons in gonadectomized males implanted with either testosterone (T) or blank (Bl) capsules, and exposed to females or not. A main effect of hormone treatment was detected in the AMY.

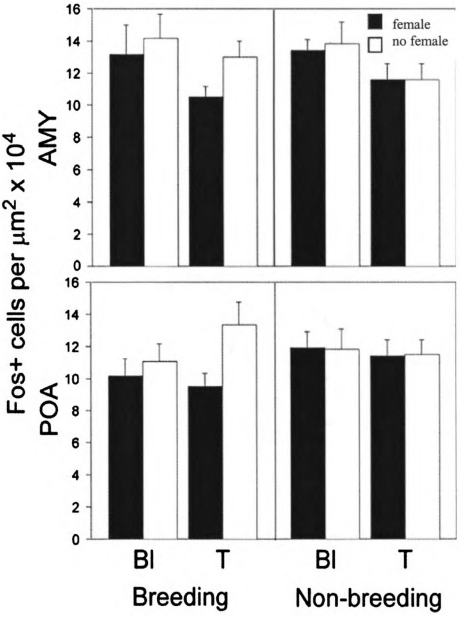


Figure 13. Photomicrographs of c-fos expression in the AMY of males given testosterone (top) or blank (bottom) capsules during the breeding season. OC = optic chiasm. The 100μm x 100μm box depicts the area of the AMY that was analyzed. Scale bar = 100μm

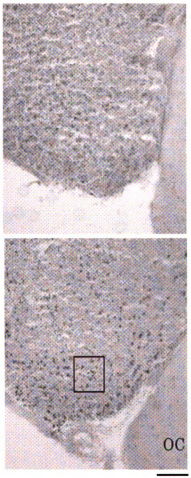


Figure 14. Relationships between courtship behaviors (dewlap extensions) and the density of c-fos+ cells in the POA. Black circles represent testosterone-treated males exposed to females in the breeding season and gray circles indicate all other males exposed to females.

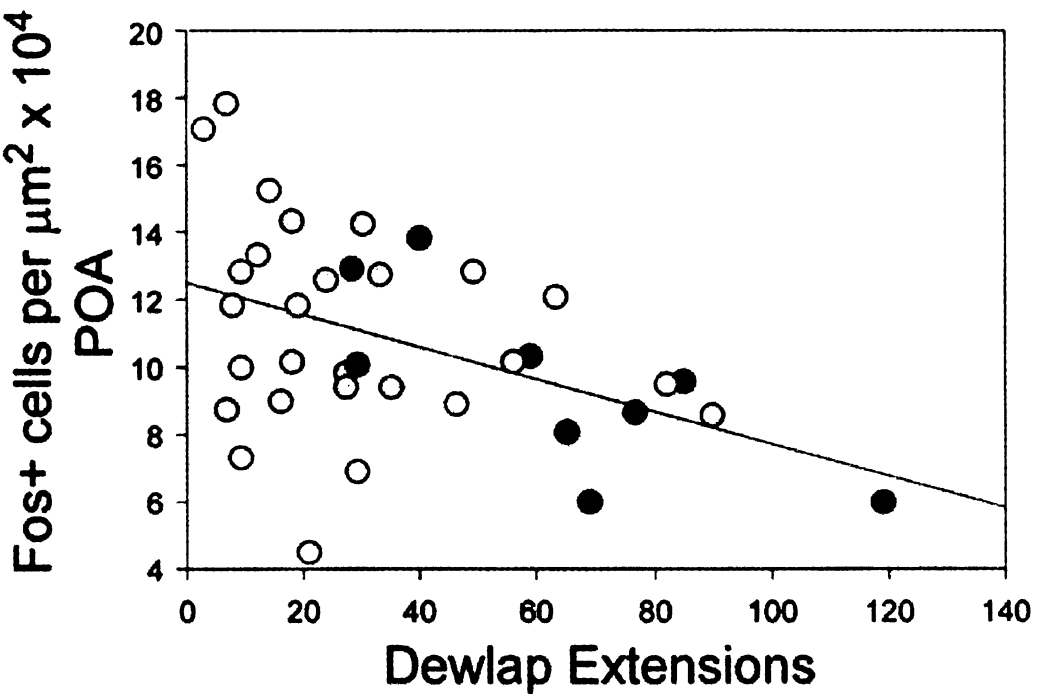
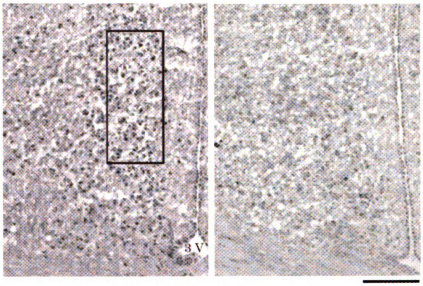


Figure 15. Photomicrographs of c-fos immunoreactivity in the POA of males displaying high (left; 119 dewlap extensions) and low (right; 33 extensions) levels of behavior. The 250μm x 100μm box depicts the area of the POA that was analyzed. 3V = third ventricle. Scale bar = 100μm.



CHAPTER 5 : DISCUSSION

The studies in this dissertation have investigated relationships across multiple sites regulating behavioral function (forebrain, muscle and motoneuron) in manipulated and gonadally intact adult males between seasons. They confirmed that male sexual behavior is enhanced by testosterone (T), breeding environmental (BS) conditions and female presence (Crews et al., 1978; Greenberg and Nobel, 1944; Jenssen et al., 2000; Lovern et al., 2004b; Neal and Wade, 2007; O'Bryant and Wade, 1999; Rosen and Wade, 2000; Wade, 2005; Winkler and Wade, 1998), and that males are behaviorally more sensitive to T and female exposure in the BS than non-breeding (NBS) season (Neal and Wade, 2007). In parallel, T enhances preoptic area (POA) and amygdala (AMY) soma size as well as copulatory muscle (RPM) fiber size, and the hormone decreases neuronal activity in the AMY (possibly of inhibitory neurons; (Holmes and Wade, 2004b; Neal and Wade, 2007). Like the effect of T on behavior, AMY soma and copulatory muscle morphology are increased more in the BS than NBS (Neal and Wade, 2007).

Similar effects of T and season on behavior and morphology are observed in sexually dimorphic forebrain regions across a wide range of vertebrates, as well as in copulatory motoneurons and muscles of mammals and courtship motoneurons and muscles in fishes, birds and frogs (Alvarez-Borda and Nottebohm, 2002; Brahic and Kelley, 2003; Breedlove and Arnold, 1980; Breedlove and Arnold, 1981; Breedlove and Arnold, 1983; Brenowitz, 1997; Crews et al., 1993; Forger and Breedlove, 1987; Hegstrom et al., 2002; Knapp et al., 1999; Li et al., 2000; Nottebohm et al., 1986; Panzica et al., 1996; Paredes, 2003; Rasika et al., 1994; Romeo et al., 2001; Sakata et al., 2003;

Smith, et al., 1997c; Thompson and Brenowitz, 2005; Thompson and Adkins-Regan, 1994; Tobias and Kelley, 1987; Tramontin and Brenowitz, 2000; Tramontin et al., 1999; Wade and Crews, 1991; Wade et al., 1993; Wood, 1996; Wood and Swann, 2000). Thus, some of the effects of T are quite similar in anoles and other vertebrates. However, the lack of effects of adult T on courtship or copulatory motoneuron soma size and courtship muscle fibers size differs from a number of other model systems. It is also possible that some undetected morphological plasticity may exist in response to T and/or season. For example, T increases dendritic arborization in rats compared to those given androgen blockade (Rand and Breedlove, 1995).

Also, in contrast to some of the species discussed above, T decreases neuronal activity in the anole forebrain independently of seasonal cues or female presence. For example, in starlings, c-fos expression in the POA and song nuclei positively correlates with behavior in response to female presence during the BS when T levels are high, but not in the NBS when T declines (e.g., songbirds and rodents, Heeb and Yahr, 1996; Heimovics and Riters, 2005; Heimovics and Riters, 2006; Kollack-Walker and Newman, 1995; Kollack-Walker and Newman, 1997; Mello, 2002; Schwab et al., 2004). However, this effect in anoles parallels the negative correlation between neuronal activity (indicated by the density of c-fos expressing cells) and the number of dewlap extensions in the POA (Neal and Wade, in press). This relationship is mainly observed in T-treated males in the BS, and is consistent with the idea that T decreases the activity of neurons that normally inhibit reproductive behaviors. Such a result has been detected in gerbils; c-fos is expressed in GABA neurons after mating (gerbils, Simmons and Yahr, 2003).

Individual variations in behavior are correlated with some aspects of anole reproductive morphology in a manner similar to other species. For example, dewlap muscle fiber and AMY soma size are positively correlated with the rate of dewlap extensions in unmanipulated male anoles during the BS (Neal and Wade, in press) similar to findings in rodents, rams, rats and birds in which relationships between individual variations in behavior and forebrain and courtship and copulatory neuromuscular structures have also been documented (e.g., Alexander et al., 2001b; Bass and Baker, 1990; Bass and Marchaterre, 1989; Bass and Zakon, 2005; Bernard et al., 1996; Brantley et al., 1993; Breedlove, 1997; Prince et al., 1998). However, while most of these correlations are positive, a negative relationship between behavior and morphology exists in the SNB. Specifically, spinal motoneurons and copulatory muscle fibers are smaller in rats who copulated compared to those who did not (Breedlove, 1997; Raouf et al., 2000).

In systems in which androgens are important for regulating functional and morphological changes, androgen receptors (AR) likely play a major role. This is particularly true in green anoles, as T (and not one or more metabolites) is the primary activator of male sexual behavior (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). They are present in all of the reproductive structures studied (forebrain, courtship and copulatory neuromuscular systems) in the anole and in some birds, mammals, frogs and fish, in which adult T enhances morphology in part by up-regulating AR (Arnold and Breedlove, 1985; Bass and Marchaterre, 1989; Bass and Zakon, 2005; Breedlove and Arnold, 1980; Breedlove and Arnold, 1981; Breedlove and Arnold, 1983; Cooke et al., 1998; Forger et al., 1992;

Forger et al., 1996; Freeman, et al., 1995; Gurney, 1981; Gurney, 1982; Kay et al., 1999; Kelley, 2004; Kelley et al., 1988; Lu, et al., 1998; Meek, et al., 1997; Monks, et al., 2004; Park et al., 2002; Perez and Kelley, 1996; Potter et al., 2005; Rand and Breedlove, 1995; Wade and Buhlman, 2000; Wetzel and Kelley, 1983). However, among neuromuscular structures in the anole, AR expression is increased with T treatment only in the copulatory muscles (RPM, Holmes and Wade, 2005b; Rosen et al., 2002). Therefore, increasing AR expression may be one mechanism through which T enhances RPM fiber size and the fact that this effect does not occur in the dewlap muscle may provide a reason why this structure does not appear plastic in adulthood. This mechanism of T increasing morphology through AR expression may also underlie increases in POA and AMY soma size and decreases in neuronal activity following T treatment. However, it is currently unknown what effects T has on AR expression in the POA and AMY. That work is currently being done.

While morphological plasticity and neuronal activity can be induced in some tissues with a relatively high dose of exogenous T, studying individual differences in morphology in unmanipulated animals revealed that routine seasonal changes in circulating T levels are probably not sufficient to induce morphological change in any of the neuromuscular structures. Instead, it is possible that the differences between the sexes on multiple levels, including neuron soma size and muscle fiber size, and across males (AMY and dewlap muscle) are created by differences in T levels during development. In particular, a large sex difference in ceratohyoid fiber size exists in adulthood, and the muscle is sexually dimorphic by post hatching day 75 (O'Bryant and

Wade, 2001). This sexual dimorphism may be due to different levels of T exposure beginning around post hatching day 30 (Lovern, et al., 2004a).

A particularly interesting feature of the green anole is that both courtship and copulatory motoneurons differentiate far later than their muscle counterparts. They do not differentiate until sometime after post-hatching day 90 (Holmes and Wade, 2005a; Lovern et al., 2004a; O'Bryant and Wade, 2001), which makes a mechanism similar to the one involved in sexual differentiation of the rodent SNB unlikely. In that case, trophic factors from muscles are critical for motoneuron survival (reviewed in Sendtner, et al., 2000). However, in the anole, the muscles regulating dewlap extension are present but dimorphic at least 25 days before the motoneurons are. Even more strikingly, the copulatory muscles are completely absent in females prior to hatching, so more than 90 days before the motoneurons differentiate. The mechanisms of motoneuron differentiation (as well as the specific timing) are completely unknown, but one possibility is that differences in levels of use as animals enter their first breeding season are involved.

It is also currently unknown when the POA and AMY are sexually differentiated during development in the anole, but it is possible that T masculinizes these regions. For example, T (aromatized to estrogen) mediates sexual differentiation of the POA in rats and ferrets around the time of birth (reviewed in Morris, et al., 2004). It is therefore possible that individual differences in morphology and neuronal activity seen in adulthood are the result of individual variations in T levels during development. Steroid receptor co-activators may also influence responsiveness of forebrain and courtship and

copulatory neuromuscular structures during different developmental time points and/or between seasons in adults.

When evaluating the present studies, it is important to consider the implications of what increased morphology confers on a structure. For example, axonal diameter, conduction velocity and dendritic arbors positively correlate with soma size in mammals and birds (Airey and DeVoogd, 2000; DeVoogd and Nottebohm, 1981; Kernell and Zwaagstra, 1981; McPhedran, et al., 1965; Smulders and DeVoogd, 2000; Wuerker and Henneman, 1963). Also, motoneurons with larger somas have less input resistance than smaller neurons (Henneman, et al., 1965). Therefore, a larger soma may be more likely to facilitate a response and thus result in greater integration of environmental stimuli and hormonal status in the POA and AMY for the expression of behaviors. Larger muscles are associated with greater force and speed than smaller muscles (Sale, 1987), suggesting that perhaps studly males with larger dewlap muscle fiber size are able to extend their dewlaps quicker and/or maintain dewlap extensions for longer. It is equally possible that these individuals have larger dewlaps, such that more force (and larger muscles) would be needed to extend it. This would be easy to test by measuring dewlap size in males with a range of behavioral variability.

The present work has replicated and expanded previous investigations on the courtship and copulatory neuromuscular systems and the motivational forebrain areas involved in male green anole reproductive behavior. It now remains to investigate other factors that may affect behavior and morphology. The anole genome is currently being sequenced, and it will be important to examine the forebrain and courtship and copulatory

systems at the molecular level to fully understand the mechanisms underlying adult behavioral and morphological plasticity.

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