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THE ROLE OF TESTOSTERONE IN SEXUAL DIFFERENTIATION AND ADULT PLASTICITY IN A LIZARD: THE COPULATORY NEUROMUSCULAR SYSTEM OF GREEN ANOLES (*ANOLIS CAROLINENSIS*)

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By

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ABSTRACT

THE ROLE OF TESTOSTERONE IN SEXUAL DIFFERENTIATION AND ADULT PLASTICITY IN A LIZARD: THE COPULATORY NEUROMUSCULAR SYSTEM OF GREEN ANOLES (*ANOLIS CAROLINENSIS*)

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The neuromuscular system controlling copulation in green anoles (*Anolis carolinensis*) is a powerful system for investigating sexual differentiation and adult plasticity in the nervous system. Green anoles are native to the southeastern United States, they are strict seasonal breeders (i.e. they only breed during long days with warm temperatures), and their copulatory neuromuscular system is remarkably sexually dimorphic. Adult males possess two intromittant copulatory organs called hemipenes, each independently controlled by two muscles and separate populations of associated motoneurons. Adult females do not possess any of these structures. In male anoles the gonads regress during the transition to winter conditions (shorter days and cooler temperatures) and this regression results in decreased secretion of testosterone (T), resulting in the cessation of reproduction. The present experiments clearly demonstrate several conclusions about T action on the anole copulatory system. 1) Seasonal cues have limited effects on neuromuscular morphology of gonadally intact males. 2) Exogenous T has trophic effects on copulatory muscles and hemipenes in adulthood. 3) The soma size of motoneurons is not responsive to T in adulthood, demonstrating a dissociation between the periphery and central nervous system. 4) T treatment regulates expression of the androgen

receptor (AR) protein in a manner parallel to its morphological effects. 5) T has trophic effects on the periphery, but not motoneurons, in juvenile males; no effects are seen in juvenile females, suggesting sexual differentiation is complete and non-reversible at this developmental stage. Finally, 6) T or the non-aromatizable androgen dihydrotestosterone rescues this system in female embryos, and administration of estradiol causes regression of the system in male embryos. These results demonstrate that T is involved in both the development and maintenance of the lizard copulatory system. Comparing these data to T effects in dewlap neuromuscular systems, which is involved in male courtship behavior, reveals an intriguing selective sensitivity to T in green anoles. T has very specific periods of action, as well as particular targets, even between two sexually dimorphic neuromuscular systems in the same animal. Understanding additional morphological and physiological parameters that respond to T in both of these systems will further help identify the specific mechanisms via which T mediates reproductive behaviors, and the neural circuits that underlie them, in this species.

To Ashley and my family,
with endless love and gratitude.

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KEY TO ABBREVIATIONS

AmbIX/VIImv	ventral motor nucleus of the facial nerve
AmbX	vagal portion of the nucleus ambiguus
ANOVA	analysis of variance
AR	androgen receptor
BC	bulbocavernosus muscle
BS	breeding season
BSX	breeding season with regressed testes
CF	caudifemoralis
CH	ceratohyoideus muscle
CT-FITC	cholera toxin-B subunit conjugated to fluorescein
DAPI	4',6-Diamidino-2-Phenylindole
DHT	dihydrotestosterone
DY	diamidino yellow
E	estradiol
EOD	electric organ discharge
FB	fast blue
GDX	gonadectomized; castrated
ID	inner diameter
LA	levator ani muscle
NBS	non-breeding season
nXIIts	tracheosyringeal portion of the hypoglossal nucleus
OD	outer diameter
PBF	phosphate buffered formalin
PBS	phosphate buffered saline
RIA	radioimmunoassay
RPM	retractor penis magnus muscle
SC	cloacal sphincter
SNB	spinal nucleus of the bulbocavernosus
SVL	snout-vent length
T	testosterone
T17-S1	spinal segments trunk 17-sacral 1
TPN	transversus penis muscle

INTRODUCTION

I) Sexual dimorphisms in brain and behavior

Men and women are different. This truism is widely understood, even by children at a very young age. We understand that males and females, humans or otherwise, are often different in the way they look and in the way they behave. That is, there are *sexual dimorphisms* between males and females in both anatomy and behavior. Darwin (1871) described three types of sex-related traits that differ between the sexes: primary, secondary, and 'ecological'. Primary sex traits are defined as those structures directly related to reproduction (*i.e.* the gonads, the associated ducts and copulatory organs). Secondary sex traits are anatomical features that indirectly participate in reproduction by increasing the probability of copulation, including armaments and ornamentations that are involved in attraction of or competition for mates. Ecological sex traits may have no bearing on reproductive fitness, but arise as a consequence of males and females of a species experiencing different ecological pressures and/or occupying different niches (Andersson 1994). For example, in *Centurus striatus* (a woodpecker species) males and females have very dimorphic bill morphology, which reduces food competition between the sexes and ultimately expands the food niche of the breeding pair (Ligon 1968; Selander 1966; as described in Andersson 1994). Although Darwin's concept of sexual dimorphism elegantly categorizes gross morphology and behavior, it is not always clear how it applies to the nervous system. Therefore, understanding neural dimorphisms in simple behavioral systems (e.g. neuromuscular systems, see below) that are

ecologically relevant, may help elucidate relationships among dimorphisms in brain, behavior, reproduction, and ecology.

Despite the ubiquity of described gross anatomical and behavioral sex differences throughout the animal kingdom, it is only relatively recently that dimorphisms in the nervous system have received widespread attention. In fact, dimorphisms have been identified in the nervous systems of all vertebrate classes. Furthermore, within the nervous system, sex differences can occur at a variety of levels, ranging from molecular to electrophysiological to gross morphological. Of great interest is how these sex differences develop, how they are maintained in adulthood, and how they relate to behavior. Given the strong connection that neural dimorphisms often share with reproductive behavior and strategy, it is also not surprising that there is a role for reproductive hormones in the development and maintenance of sex differences in the central nervous system.

II) Steroid hormone effects on the nervous system: organization vs. activation

The classic template for describing the effects of gonadal hormones on brain and behavior is to categorize effects as either a) organizational or b) activational. This dichotomy, first proposed by Phoenix and colleagues (1959), describes how steroid hormones may act early in development to permanently *organize* neural circuits or may act in adulthood to transiently *activate* behavior. The criteria that distinguish these effects are the duration of the effect, the life stage during which the hormone acts, and whether the effect occurs during a

critical period (Phoenix et al., 1959; Arnold & Breedlove, 1985). While the heuristic is very useful, a growing body of literature reveals multiple exceptions to these rules (see Arnold & Breedlove, 1985). Nevertheless, there are substantial empirical data to support the hypothesis that testicular steroids (androgens) in particular permanently organize the developing nervous system. However, the emerging story is anything but simple. Testosterone (T) is the primary androgenic steroid hormone that is secreted by the testes. However, T per se is often not *directly* responsible for sexual differentiation and/or adult neuroplasticity. T often serves as a pro-hormone when metabolism to 5-α-dihydrotestosterone (DHT; via reduction by the 5-α-reductase enzyme) or aromatization to estradiol-17β (E; via the aromatase enzyme) is required for biological effects (Nelson, 2000).

III) Neuromuscular systems and structure/function relationships

Because dimorphisms in the nervous system are often directly or indirectly related to behavior, it is valuable to understand both how they develop (sexual differentiation) and how they may change in adulthood (adult plasticity). Neuromuscular systems are comprised of spinal or brainstem motoneurons and their target musculature. Therefore these systems are particularly conducive to the study of both sexual differentiation and adult plasticity due to the direct link between neuromuscular activity and behavior. Given that the behavioral functions of neuromuscular systems are often obvious (Breedlove et al., 2002), the adaptive significance of dimorphisms is often obvious, particularly in systems directly involved in reproduction. Indeed, it has been proposed that

understanding the mechanisms of steroid hormone action on neuromuscular systems will assist with the understanding of hormone action on more complicated neural systems (Breedlove et al., 2002).

IV) Comparative approaches for investigating sexually dimorphic neuromuscular systems

In order to understand general principles of both sexual differentiation and adult neuroplasticity, one must use a comparative approach. A brief review of what is presently known about sexually dimorphic neuromuscular systems reveals that their development and maintenance involve disparate mechanisms among vertebrate groups. Yet similarities between model systems do exist, suggesting that some mechanisms of hormonal action on differentiation and plasticity may be more conserved than others.

Communication in fish

A vocalizing (sonic) species of fish (Midshipman fish, *Porichthys notatus*) has two classes of sexually mature males – Type I and Type II (Brantley et al. 1993a). These two classes have different reproductive strategies and can be distinguished by a number of traits. Type I males are larger (than Type II males or females), build nests, guard eggs, and generate vocalizations or "hums," which are generated by the contraction of a single pair of muscles attached to the lateral walls of the swimbladder. The Type I male vocalizations are performed during the breeding season, to attract gravid females to their nests. In contrast, Type II males sneak-spawn, and neither Type II males nor females perform these

"hums". The frequency of the sound generated is determined by the synchronous activity of central motoneurons that are located in the caudal brainstem. Sexual dimorphisms are present at several levels of this system including number and size of motoneurons, as well as total muscle and muscle fiber size (Bass and Marchaterre 1989a; Bass and Marchaterre 1989b; Bass and Baker 1990). In all of these features, Type I males are larger than either Type II males or females (which are comparable to each other).

While sonic muscle size is larger in *adult* Type I males, it is not dimorphic in juveniles (Bass and Marchaterre 1989a) which is likely due to the relatively low levels of androgen at this developmental stage (Brantley et al. 1993a). In fact, this muscle is androgen-sensitive in all 3 phenotypes (Type I males, Type II males, and females), whereby androgen exposure increases muscle mass and fiber structure toward the Type I male phenotype (Brantley et al. 1993b). Type I and Type II males have different androgen profiles: 11-Ketotestosterone is the predominant androgen in Type I males while T is the most prevalent androgen in Type II males (Brantley et al. 1993c). Androgen profiles in Type II males more closely resemble that of females. While additional data are required to conclusively state which particular androgens are responsible, it is likely that there is a role for androgen in the development and/or maintenance of these inter- and intra-sexually dimorphic structures.

Another group of teleosts, weakly electric fish, communicate via an electric organ discharge (EOD). Production of the EOD occurs via activation of a central motor pathway that excites an electric organ, comprised of modified muscle cells,

located in the tail. Several species use sexually dimorphic EOD frequencies to recognize the gender of a conspecific (Dunlap et al. 1998), but the direction of this behavioral dimorphism varies across species. Similar to the sonic system in midshipman, the electric organ system is functionally responsive to androgens and estrogens whereby treatment with E will feminize the signal, while non-aromatizable androgens masculinize it. Effects of androgens on morphology of the system are species-specific whereby some species exhibit larger electrocytes in response to T while others do not (e.g. Mills et al. 1992). Therefore, species differences in the direction of functional and morphological dimorphisms can be explained by species-specific responses to these steroids (see Zakon & Dunlap, 1999).

Vocalization in frogs

Adult male African clawed frogs (*Xenopus laevis*) use vocalizations to court females, and the neuromuscular system underlying this behavior is sexually dimorphic at multiple levels. Motoneurons located in the caudal medulla send axons through cranial nerves IX-X to the larynx (Wetzel et al. 1985). These axons synapse on the laryngeal dilator muscle, and contraction of this muscle causes vocalizations to be produced (Tobias and Kelley 1995). The motoneurons are larger and more numerous in males than in females (Hannigan & Kelley 1981; Kelley & Fenstemaker 1983). The laryngeal muscle is also dimorphic in fiber type composition (males have one fiber type while female muscles are heterogeneous), as well as fiber size and number (favoring males; Sassoon &

Kelley 1986). Also, the larynx is 2-3 times larger overall in males than in females due to increased muscle and cartilage mass (Sassoon & Kelley 1986).

Production of male specific courtship vocalizations depends on gonadal androgens (T or DHT) but not E (Wetzel & Kelley 1983), although treatment with T will *not* induce a masculine call in adult females (Hannigan & Kelley 1986). This lack of behavioral plasticity in adult females is likely a result of androgen-regulated masculinization during development (Kelley 1986). Either T or DHT (but not E) is capable of inducing laryngeal myogenesis in juveniles of either sex (Sassoon et al. 1986). Furthermore, while the number of motoneurons is not plastic in adulthood, soma size is - androgen treatment can shift females in the masculine direction (Hannigan & Kelley 1983). Of particular interest is that implantation with male testicular tissue is more successful at masculinizing females than prolonged T treatment. Adult females can produce male typical vocalizations as a result of these testicular implants but the effect is more striking when implantation occurs while the females are still juveniles (Watson & Kelley 1992), suggesting that complete masculinization requires exposure during a critical period and/or testicular secretions other than T.

Vocalization in song birds

A significant body of literature exists on mechanisms regulating sexual differentiation and adult plasticity in the adult passerine brain. In particular, the zebra finch is a popular model for investigating hormonal and non-hormonal effects on sexual differentiation (see Wade 2001 for review). Despite this fact,

surprisingly little work has been done on the neuromuscular system that actually controls the output of song. This system consists of motoneurons located in the tracheosyringeal portion of the hypoglossal nucleus (nXIIts) and their target muscles: two of which are the ventralis and dorsalis muscles of the syrinx. Dimorphisms exist at both the neural and muscular levels of this system. nXIIts volume, syrinx mass and both dorsalis and ventralis muscle fiber size are larger in males than females (Nottebohm and Arnold 1976; Springer and Wade 1997; Wade et al. 1999; Wade and Buhlman 2000). Treatment with T in adulthood can increase syrinx weight and muscle fiber size in adult females, although the effects of T on nXIIts volume, motoneuron soma size or number are variable (Arnold 1980; Gurney 1981; Wade and Buhlman 2000). Similarly, T treatment slightly increases syrinx weight and DHT increases nXIIts motoneuron soma size in juvenile females (Wade et al. 2002). Further supporting a role for androgens, adult males have greater androgen receptor (AR) mRNA expression than females in syrinx muscle and cartilage (Veney and Wade, 2004), although no sex difference in AR mRNA was detected at days 3, 10, and 17 post-hatching (Veney and Wade, 2005). However, E may also mediate the differentiation of some components of this system. E appears to actively feminize the syrinx as E-treatment decreases muscle fiber size and overall syrinx weight in juvenile males (Wade et al., 2002).

Copulation in mammals

Perhaps the best characterized sexually dimorphic neuromuscular system is the spinal nucleus of the bulbocavernosus (SNB) system in rodents. This system is responsible for controlling penile reflexes that are required for successful fertilization. Motoneurons in the lower lumbar spinal cord project to the bulbocavernosus (BC) and levator ani (LA) muscles that attach to the base of the penis. In adult male lab rats, SNB motoneurons are larger and more numerous than in females, and the BC/LA musculature is either vestigial or absent in adult females (Breedlove and Arnold 1980). These dimorphisms develop shortly after birth as both males and females are born with BC/LA muscles attached to the base of the penis and/or clitoris and these muscles are already innervated by SNB motoneurons (Rand and Breedlove 1987). During this perinatal period the BC/LA muscles atrophy and the corresponding motoneurons die in females (Nordeen et al. 1985).

The development and maintenance of this system is remarkably sensitive to gonadal androgens. Perinatal T treatment can rescue SNB motoneurons as well as the BC/LA musculature in females, and perturbation of androgen signaling (via pharmacological manipulation or castration) causes regression of these structures in developing males (Breedlove & Arnold 1983a & b). These effects are due to the biological activity of the androgen receptor, which was elegantly shown by the use of androgen insensitive rats. Testicular feminization mutation (tfm) results in genotypic (XY) males possessing non-functional

androgen receptors. As such, these animals are insensitive to circulating levels of androgen (either endogenous or exogenous) and develop a female phenotype (Breedlove & Arnold 1981).

The SNB system is also sensitive to circulating steroids in adulthood. Castration of adult males results in decreased SNB motoneuron soma and nucleus size, dendritic arborization, neuromuscular junction (NMJ) size and BC/LA muscle mass (e.g. Balice-Gordon et al 1990; Breedlove and Arnold 1981; Rand and Breedlove 1995). All of these effects can be reversed by T replacement. T acts directly on the BC/LA muscles to increase muscle fiber size and motoneuron dendrites but acts on the motoneurons themselves to influence soma size.

The SNB system can also show significant naturally occurring (and therefore potentially functionally relevant) adult plasticity in seasonally breeding species (e.g. the white-footed deer mouse, *Peromyscus leucopus*, and the Siberian hamster, *Phodopus sungorus*). Specifically, SNB motoneuron somata and nuclei size, neuromuscular junction size, and BC/LA muscle mass are larger in breeding males (Forger & Breedlove 1987; Hegstrom and Breedlove 1999; Hegstrom et al. 2002). The alterations in soma and nucleus size, as well as muscle mass and fiber size, are a result of corresponding changes in circulating androgens (decreased in non-breeding males due to regression of the testes). However, T is not directly responsible for all changes in this system; for example, NMJ size appears to be sensitive to photoperiod independent of circulating levels of T (Hegstrom et al. 2002).

V) The green anole as a reptilian model for neuroendocrine investigations of neuromuscular systems

Recent work, primarily in the laboratory of Juli Wade, has focused on establishing the green anole (*Anolis carolinensis*) as a reptilian model for investigations of sexual differentiation and adult neuroplasticity. Green anoles differ from more traditional laboratory species in that they possess both a sexually dimorphic *copulatory* neuromuscular system and a dimorphic neuromuscular system involved in *courtship*. As such, they provide a unique opportunity to compare sexual differentiation and adult plasticity of two dimorphic neuromuscular systems within the same species.

The model system: Anolis carolinensis

One advantage of the use of green anoles as a comparative model system for neuroendocrine research is that there is a remarkable amount of field and laboratory data describing the ecology, behavior and physiology of this species. The social organization is polygynous with males competing for and establishing large territories that encompass the smaller territories of multiple females (Ruby 1984; Jenssen et al. 1995a; Jenssen and Nunez 1998). Males defend their territories in an attempt to maintain exclusive reproductive access to these females. Larger males establish and defend larger territories and, as such, have access to more females; smaller males may not establish a territory at all, and instead become "floaters" in the habitat trying, to evade detection by other males.

Interestingly, precopulatory mate choice by females is not evident in this species. Females establish their territories independent of male location and then mate with the male that establishes the overlapping territory (e.g. Jenssen et al. 2001). Despite the apparent lack of direct female mate choice, males do court females by performing a suite of head-bobbing displays coupled with the extension of a bright red throat fan, called a dewlap. If the female is receptive she will remain stationary (often bobbing her head in return but *not* extending her dewlap) while the male approaches and mounts her, biting the back of her neck while clasping her torso with his forelegs. Intromission occurs when the male flips his tail under that of the female and inserts one of his two copulatory organs (called hemipenes) into the female cloaca. Copulation can last from less than five to more than sixty minutes (Greenberg and Noble 1944; Jenssen and Nunez 1998).

Reproduction in this species is regulated by both environmental and physiological conditions. Green anoles are strictly seasonal breeders, reproducing in the field from about April to July when days are relatively long and temperatures are relatively high. T levels in males are about twice as high during the breeding season as during the non-breeding season (Lovern et al. 2001). This T is important for the expression of male sexual behaviors, as castration decreases these behaviors while T can prevent or reverse this effect (Adkins and Schlesinger 1979; Crews et al. 1978; Mason and Adkins 1976; Rosen and Wade 2000; Winkler and Wade 1998). Males housed in non-breeding conditions in the

laboratory do not exhibit copulatory behavior, even if treated with high levels of T (O'Bryant and Wade 2002a).

The dewlap neuromuscular system

Both the dewlap and the neuromuscular system controlling its extension are sexually dimorphic (Jenssen et al. 2000; O'Bryant and Wade 1999; O'Bryant and Wade 2002b; Wade 1998). The dewlap system consists of paired ceratohyoid muscles located in the throat that are innervated by motoneurons in the caudal brainstem via the ramus pharyngo-laryngeus nerve (Wade 1998). When they contract, the ceratohyoid muscles cause the cartilage to bow out to extend the dewlap from the ventral surface of the neck. Both male and female anoles possess and use dewlaps, but male dewlaps are about 7-fold larger in surface area and are used more frequently than those of females (Greenberg and Noble 1944; Jensson et al. 2000; Nunez et al. 1997). Furthermore, both males and females will extend their dewlaps in aggressive encounters, but only males use the dewlap in courtship (Greenberg and Noble 1944). Dimorphisms are present at multiple levels of this system including motoneuron soma size, nerve cross-sectional area, neuromuscular junction (NMJ) size, cartilage length and muscle fiber size, number and density (all favoring males with the exception of muscle fiber density; Wade 1998; O'Bryant and Wade 1999; O'Bryant and Wade 2002b). Interestingly, the morphology of this system is not plastic in adulthood (O'Bryant and Wade 1999); it does not vary naturally across season nor does it respond to changes in circulating levels of T (castration vs. T

replacement). This may not be surprising given that, while only males use the dewlap for courtship during the breeding season, both males and females continue to use their dewlaps across seasons for aggressive interactions (albeit at much lower frequencies during the non-breeding season).

The dewlap neuromuscular system is not sexually dimorphic at the time of hatching but is fully differentiated by adulthood. It is not yet known whether differentiation of the dewlap system is mediated by T. However, the time course of differentiation as reported by O'Bryant and Wade (2001) does parallel a developmental rise in T that occurs only in juvenile males (Lovern et al. 2001). Chapter 5 (see below) directly addresses whether T masculinizes the dewlap system during the period of its differentiation.

The hemipenis neuromuscular system

Like the sexually dimorphic dewlap system, the copulatory neuromuscular system in green anoles is dimorphic on multiple levels (Ruiz and Wade 2002). Male anoles possess bilateral copulatory organs (located in the tail) called hemipenes that are independently controlled by two muscles that work in an antagonistic manner (see Figure 1). The transversus penis (TPN) wraps over the hemipenis with the muscle fibers running in a medial to lateral orientation; when the TPN contracts the hemipenis is everted through the cloaca (Arnold 1984). The retractor penis magnus (RPM) is attached to the base of the hemipenis with the fibers running in a rostral to caudal orientation; contraction of the RPM

retracts the hemipenis back into the tail (Arnold 1984). Adult females do not possess hemipenes or either TPN or RPM muscles.

Injection of the retrograde neuronal tracer biocytin into the TPN revealed that the motoneurons projecting to this muscle are located in the pelvic region of the spinal cord in spinal segments trunk 17 and sacral 1 (T17-S1). Anoles possess 17 trunk segments analogous to thoracic and lumbar segment in mammals as well as 2 sacral segments (Ruiz and Wade 2002). The TPN motoneurons are located in the ventrolateral portion of the ventral horn and they are larger and more numerous in males than in females (Ruiz and Wade 2002).

Prospectus

Given that green anoles are seasonal breeders and we know that copulatory systems can be plastic in seasonally breeding mammals, it may be that their copulatory neuromuscular system is a novel system with which to investigate naturally occurring and functionally relevant adult neuroplasticity. The experiments described below are essential not only for the characterization of the green anole copulatory neuromuscular system *per se*, but also for the establishment of this system for comparative investigations of sexual differentiation and adult neuroplasticity both within this species (courtship vs. copulation) and across vertebrate groups. Specifically, in order to bring present understanding of this model system up to the level of other sexually dimorphic neuromuscular systems, we need to know (a) how the system is organized by characterizing motoneuron projections, (b) whether this system is plastic at

different life stages, including adulthood, and if putative plasticity is due to changes in circulating androgen levels, (c) how androgen receptors (AR) are distributed in these tissues in adulthood, and (d) when this system differentiates and if differentiation is caused by androgens. Taken together, these experiments will elucidate the role of androgen in the copulatory neuromuscular system of male green anoles and will help determine whether mechanisms of steroid action on copulatory systems (both in development and adulthood) are conserved across species.

The experiments described in **Chapter 1** identify the location of RPM motoneurons and characterize the relative distribution of TPN and RPM motoneurons within spinal cord segments T17-S1. The experiments in **Chapter 2** investigate whether the sexually dimorphic copulatory neuromuscular system is plastic in adulthood, and the role that testosterone plays. **Chapter 3** examines the distribution of androgen receptor (AR) in the copulatory tissues (including the spinal motoneurons, musculature, and hemipenes) and dewlap tissues (including muscles and cartilage) of adult males. **Chapter 4** investigates the effects of juvenile testosterone in sexual differentiation of the dewlap and copulatory systems. Finally, **Chapter 5** examines (a) the developmental time course of differentiation of the copulatory neuromuscular structures, and (b) the effects of steroid hormones on this process during embryonic development.

CHAPTER ONE

Holmes MM, Wade J (2004). Characterization of projections from a sexually dimorphic motor nucleus in the spinal cord of adult green anoles. J Comp Neurol 471: 180-187.

INTRODUCTION

Neuromuscular systems are exceptionally well suited to investigations of structure/function relationships in the central nervous system. The direct link between neural activity and muscle contraction, as well as the often obvious behavioral function of the target structure, make it easier to identify the adaptive significance of dimorphisms in these compared to more complicated neural systems (Breedlove et al., 2002). The function of structural differences is particularly evident in sexually dimorphic neuromuscular systems involved in reproductive behaviors, and examples of such systems exist in all vertebrate classes. In species that exhibit male vocal courtship behavior (including frogs, songbirds and some fish), the neuromuscular system mediating production of vocalizations contains motoneurons and/or muscles that are larger in males than females (*e.g.* Sassoon and Kelley, 1986; Simpson et al., 1986; Bass and Marchaterre, 1989a,b; Wade and Buhlman, 2000). The system underlying mammalian copulation shows striking dimorphisms in motoneuron and muscle fiber number as well as size, again favoring males (reviewed in Breedlove et al., 2002).

The green anole lizard (*Anolis carolinensis*) has similar sexually dimorphic neuromuscular systems, one involved in courtship and the other in copulation. Adult males court females by performing head bobbing displays coupled with extension of a red throat fan, called a dewlap. Females have much smaller dewlaps than males, and extend them far less frequently, only in agonistic encounters (Greenberg and Noble, 1944; Nunez et al., 1997; Jenssen et al.,

2000). The motoneurons controlling dewlap extension are in the caudal brainstem and innervate the paired ceratohyoid muscles, located in the throat (Font and Rome, 1990; Wade, 1998); contraction of these muscles causes the second ceratobranchial cartilage to bow away from the ventral surface of the throat, extending the dewlap (Bels, 1990). Similar to the systems outlined above, many components, including motoneuron somata and ceratohyoid muscle fibers, are larger in males than females (Wade, 1998; O'Bryant and Wade, 1999; O'Bryant and Wade, 2002b).

More recently, the neuromuscular system underlying copulation in green anoles has been investigated. Male squamate reptiles (lizards and snakes) each possess two penises called hemipenes. These hemipenes are located bilaterally in the tail, and only one is extended through the cloaca during each copulation. Two muscles mediate extension (transversus penis, TPN) and retraction (retractor penis magnus, RPM) of each hemipenis (Arnold, 1984). Adult female anoles do not have hemipenes or either of these muscles (Ruiz and Wade, 2002). Injection of the retrograde tracer biocytin into the TPN revealed that its corresponding motoneurons are located in segments trunk 17 and sacral 1 (T17-S1) in the caudal spinal cord. These cells are larger and more numerous in males than females (Ruiz and Wade, 2002).

Before one can appreciate the relationships between structure and function in a given neuromuscular system, one needs to understand how the motoneurons, nerves, and muscle(s) that comprise it are organized. While this work has been largely completed for other sexually dimorphic neuromuscular

systems (*e.g.* Nottebohm et al., 1976; Schroder, 1980; Bass, 1985; McKenna and Nadelhaft, 1986; Simpson et al., 1986; Wade, 1998), organization of the anole copulatory system has not yet been fully characterized. Importantly, the motoneurons projecting to the RPM must be located. The first goal of the present investigation was to find these cells in males (Study 1). Additional goals of this investigation were to identify other muscle targets for T17-S1 motoneurons and to estimate the relative proportions of T17-S1 motoneurons that project to each in both males (Study 2a) and females (Study 2b). In addition to the TPN, the caudifemoralis (CF) receives projections from T17-S1 motoneurons (Ruiz and Wade, 2002). The cloacal sphincter (SC) was also considered a likely target as SC motoneurons are located in a similar pelvic region of the spinal cord in birds (Ohmori and Watanabe, 1989; Seiwert and Adkins-Regan, 1998). Furthermore, external sphincter motoneurons are interdigitated with copulatory motoneurons and share the pudendal nerve in rats (Schroder, 1980; McKenna and Nadelhaft, 1986), and cloacal muscles receive projections via the pudendal nerve in Iguanas (Akita, 1992).

MATERIALS AND METHODS

Animals and housing

Adult (>50 mm snout-vent length) male and female green anoles were purchased from Charles Sullivan Co (Nashville, TN). Prior to the commencement of each experiment, animals were group housed (1 male with 3-6 females) in 110-liter glass aquaria (76 x 30 x 48 cm). Lizards were housed in a

14:10 hour light:dark cycle with fluorescent and full-spectrum lights, as well as a heat lamp placed on one end of each aquarium. Temperature ranged from 28°C (ambient) to 38°C (directly under heat lamp) during the day to 18°C at night. These conditions approximate those the animals experience in the spring in the field and stimulate breeding in the lab. Aquaria were sprayed daily to help maintain 70% relative humidity, and water was provided *ad libitum*. Animals were fed crickets or mealworms three times a week. Following injection of tract tracers (see below), all animals were housed individually in 21-liter glass aquaria (42 x 20 x 24 cm) and cared for as above. All procedures were approved by the Michigan State University All University Committee on Animal Use and Care and conform to NIH guidelines.

Treatment, tissue collection, and analyses

Study 1. Seven adult male lizards were anesthetized with isoflurane, placed on ice, and incisions were made in the ventral surface of the tail. The retrograde tracer Fast Blue (FB; Illing Plastics, Bergfeld, Germany; 0.5 µl at 2.5-5% in 0.9% saline) was bilaterally injected into the RPMs. Additionally, the retrograde tracer Cholera Toxin-B subunit conjugated to fluorescein (CT-FITC; List Biological Laboratories; 0.5 µl at 2% in 0.9% saline) was bilaterally injected into the TPNs of the same individuals. To determine whether the projections were ipsilateral and to confirm that there were no double-labeled motoneurons, which would indicate tracers leaking into a muscle not intentionally injected or an unusual situation of a motoneuron projecting to more than one muscle, an

additional six males were injected as above with FB into *one* RPM and Diamidino Yellow (DY; 0.5 µl at 3% in 0.9% saline; Sigma) into the ipsilateral TPN. CT-FITC and FB both label the cytoplasm of the cell (Kuypers et al., 1980; Dederen et al., 1994) whereas DY primarily labels the nucleus (Keizer et al., 1983). As such, we were better able to identify possible double-labeled motoneurons when FB was used with DY. To decrease possible contamination, excess tracer was removed with a cotton swab and gel foam prior to suturing the incision with silk.

Three days following injection, lizards were overdosed with Brevital Sodium (0.03 ml) and perfused with 0.1M phosphate buffered saline (PBS; pH = 7.4) and 4% paraformaldehyde in PBS. Spinal segments were marked with India ink to permit identification of individual segments. Cords were extracted (segments T15-S2), embedded in gelatin, post-fixed in 4% paraformaldehyde for 2.5 hours and then transferred to 20% sucrose in PBS overnight. Hemipenis, TPN, and RPM were also extracted from the rostral tail in one block (which also included the CF) and processed as above. Cross sections of all tissue were cut frozen at 30 µm into PBS. Two series of alternate sections of spinal cord were mounted onto gelatin-coated slides from 9:1 dH_2O:PBS. Two series of every fourth section of muscle/hemipenis tissue was mounted from the same solution. One series each of spinal cord and muscle/hemipenis tissue was used for fluorescent analyses (see below). They were soaked in 0.1% sodium borohydride in PBS for 10 minutes to decrease auto-fluorescence (Clancy and Cauller, 1998) and rinsed twice with PBS and once with dH_2O prior to dehydration. Alternate spinal cord sections were stained with thionin. In some cases, series of tail tissue

were stained using the trichrome method to facilitate localization of specific muscles. However, leakage of fluorescent dyes into muscles neighboring those injected was obvious even in unstained tail sections. All tissue was dehydrated, cleared in xylene and coverslipped using either DPX (for fluorescent tissue) or Permount (for thionin- and trichrome-stained tissue).

Spinal cord and muscle tissue was analyzed using a microscope (Olympus BX60) capable of simultaneous visualization of the multiple fluorochromes. Spinal segments T15 through S2 were analyzed for the presence of FB+, CT-FITC+, and DY+ motoneurons; sections containing labeled cells were compared to thionin-stained tissue in which the ink marking the segments was obvious. In addition, it was noted whether the positively labeled motoneurons were ipsilateral or contralateral to the injection, and whether any double-labeled motoneurons were detected. In all cases, muscle tissue was analyzed to confirm appropriate and accurate injection sites.

Study 2a. To identify other muscle targets for T17-S1 motoneurons, and to determine the proportions of cells projecting to each muscle, adult male lizards received a single tracer injection in one of four muscles: TPN, RPM, CF or SC. Injections were unilateral in the TPN, RPM, and CF. The SC wraps around the cloaca (Arnold, 1984) and was injected at the midline in both rostral and caudal fibers. To control for possible differences in transport efficacy between tracers, all males were injected with FB (as above). For estimation of percentages of motoneurons projecting to each muscle (see below), three males were used per muscle. While more were injected, a few were not analyzed either because

analysis of the tail tissue indicated the tracer had leaked outside the intended muscle or too much auto-fluorescence existed in the spinal cord tissue. All tissue (spinal cord and muscle) was collected and processed as described for Study 1 for animals with TPN, RPM, or CF injections. When SCs were injected, the muscle dissection included the entire cloacal region.

Study 2b. To characterize T17-S1 motoneuron projections in females, five adult female anoles received a single injection of FB into either the CF (unilateral; n=3) or the CS (n=2). Tissue processing and analyses were performed as outlined for Study 2a.

For all animals in Study 2, FB+ neurons were counted in spinal segments T15-S2. All cells labeled following TPN, RPM, and CF injections were unilateral, so total counts from the alternate sections were multiplied by two to provide an estimate for one side of the spinal cord. With SC injections, labeled cells were detected on both sides of the cord, so they were compared to the total number of thionin-stained motoneurons found bilaterally; the left and right SC counts were then averaged to provide an estimate for one side of the spinal cord. To avoid double counting of cells across sections, motoneurons were assessed using the physical dissector technique (as in Ruiz and Wade, 2002; Gunderson, 1986), whereby cells were counted if the nuclei come into focus and disappear within a given section. This technique was not used for counting of fluorescent motoneurons as their nuclei are not always identifiable due to the homogenous nature of the FB label. However, double-counting of FB+ motoneurons was

unlikely given that cells were counted (a) in alternate 30μm sections (and diameter of these motoneurons is approximately 20μm) and (b) only if they were large and bright (i.e. not cell fragments or split nuclei). As with Study 1, muscle tissue was analyzed to confirm appropriate and accurate injection sites. For each animal, the FB+ total was divided by the total motoneuron count to provide an estimated percentage of the number of T17-S1 motoneurons that project to that muscle. In order to confirm that T17-S1 motoneurons were sexually dimorphic in number (Ruiz and Wade, 2002) in the present sample, total motoneuron counts (from thionin stained tissue) were compared between males (n=12) and females (n=5) using an unpaired t-test.

Fluorescent photomicrographs were obtained with an Olympus 35mm camera and Kodak Elite Chrome 400 film for color slides. Images were then digitally scanned (Polaroid Sprint Scan 35 Plus). Nissl photomicrographs were obtained using a Q Imaging MicroPublisher 5.0 digital camera. All images were sized, compiled, and labeled in Adobe Photoshop 7.0. Brightness and/or contrast were adjusted slightly as necessary to produce images matching those visible through the microscope. No other types of modifications were made.

RESULTS

Study 1. Injecting FB into the RPM and either CT-FITC or DY into the TPN revealed that TPN and RPM motoneurons are interdigitated in the T17-S1 motoneuron nucleus (Figure 1). Positively labeled motoneurons (FB, CT-FITC, and DY) were located throughout the rostral-caudal extent of T17 and S1 but

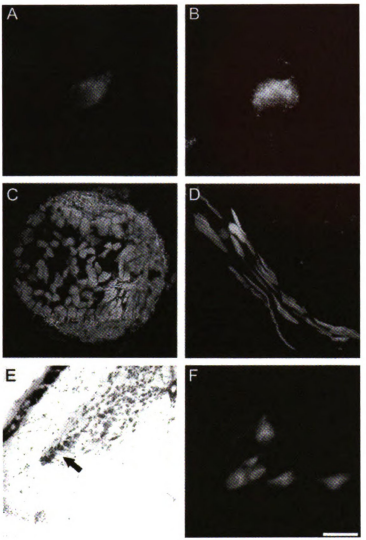

Figure 1. Photomicrographs of muscles and motoneurons from adult male anoles labeled with fluorescent tract tracers. (A) FB+ motoneuron and (C) corresponding labeled fibers from FB-injected RPM. (B) CT-FITC+ motoneuron and (D) corresponding labeled fibers from CT-FITC-injected TPN. Photomicrographs (A-D) were taken from the same animal and the motoneurons were located in adjacent tissue sections (in spinal segment T17). TPN and RPM muscle fibers run perpendicular to each other, so cross-sections of the tail produce cross-sections of RPM fibers and longitudinal sections of TPN fibers. (E) Photomicrograph of a coronal section through spinal segment T17 of an adult male anole. The India ink used to mark each segment is visible on the external surface of spinal cord, on the left side of the photomicrograph, and the central canal is visible in the top right corner. T17 motoneurons are located in the ventral horn (identified with arrow). (F) Fluorescent-labeled cells on one side of an adjacent section to that depicted in panel (E), demonstrating FB+ and DY+ motoneurons in T17. Panels (E) and (F) are from a different animal than panels (A-D). Scale bar = 10μm for (A) and (B); 95 μm for (C-E); 20μm for (F).

were not found in any other spinal segment (T15, T16 or S2); no labeled cells were found outside of the ventral horn. Unilateral injection of FB into the RPM with DY into the ipsilateral TPN (a) did not reveal any double-labeled motoneurons (Figure 1F) and (b) demonstrated that both the TPN and RPM receive only ipsilateral motoneuron projections.

Study 2a. In adult males, unilateral injection of FB into either the TPN, RPM, or CF revealed only ipsilateral labeling of motoneurons, whereas SC injections resulted in bilateral labeling of motoneurons. As with Study 1, labeled motoneurons projecting to each muscle were located throughout spinal segments T17-S1 with roughly equivalent distributions. Approximately one third of T17-S1 motoneurons each projected to the TPN and CF, whereas the remaining third projected to either the RPM or the SC. These estimates account for 96% of the motoneurons in the T17-S1 nucleus in males (Figure 2 and Table 1). The TPN counts with FB in the present study are comparable to those obtained with biocytin as reported in Ruiz and Wade (2002).

Study 2b. In adult females, unilateral injection of FB in the CF resulted in ipsilateral labeling, and SC injections resulted in bilateral labeling of motoneurons. Almost half f the T17-S1 motoneurons projected to the CF while approximately one third projected to the SC. These estimates account for 70% of the motoneurons in the T17-S1 nucleus in females (Table 1).

Comparison of the number of T17-S1 motoneurons (on one side of the spinal cord) between males (Study 2a) and females (Study 2b) demonstrates that

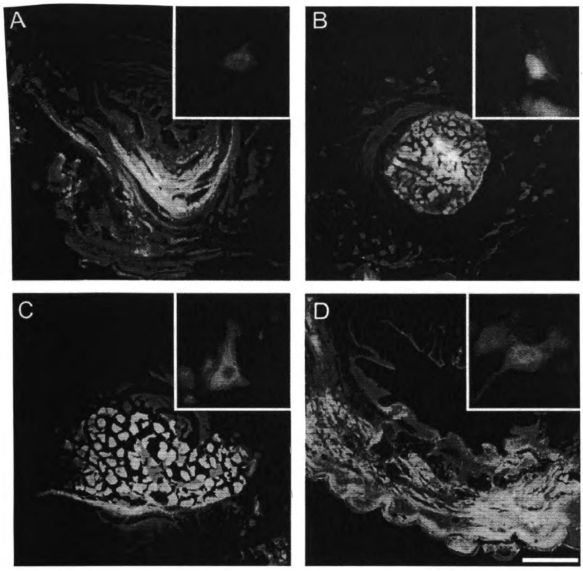

Figure 2. Photomicrographs of FB injection sites into (A) TPN, (B) RPM, (C) CF and (D) SC of adult male anoles. Corresponding FB+ motoneurons in the T17-S1 nucleus are shown in the insets. Scale bar = 360μm for (A-D) and 30μm for insets.

	TPN		RPM		CF		SC	
	Count	%	Count	%	Count	%	Count	%
Males	52	31	32	13	66	26	30	8
	90	44	40	19	82	32	46	9
	92	39	46	19	98	38	60	10
Females	N/A		N/A		54	37	64	26
					62	43	72	27
					72	49		

Table 1. Counts of fast blue-labeled motoneurons in T17-S1 following injection into *either* one transversus penis (TPN), one retractor penis magnus (RPM), one caudifemoralis (CF), or the cloacal sphincter (SC). Percentages of labeled motoneurons, based on counts from thionin-stained alternate sections, are also indicated. Means for each muscle are outlined in grey. All cells labeled following TPN, RPM, and CF injections were unilateral, so the percent labeled cells was based on thionin counts from the same side of the spinal cord. With SC injections, labeled cells were detected on both sides of the cord, so they were compared to the total number of thionin-stained motoneurons found bilaterally; SC counts represent an average of the two sides of the spinal cord. *All data points come from separate individuals (see Materials and Methods).*

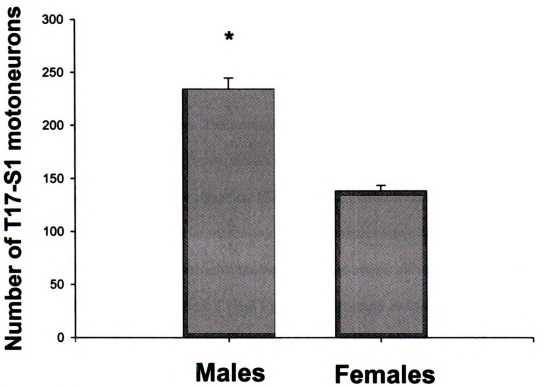

Figure 3. Total number (mean +/- SEM) of Nissl-stained motoneurons in the T17-S1 nucleus from one side of the spinal cord. Asterisk indicates a statistically significant difference between males and females.

adult males have more motoneurons in this region than adult females ($t = 5.77$; $p < 0.001$; Figure 3).

DISCUSSION

Summary

In adult male anoles, the sexually dimorphic T17-S1 nucleus is comprised of motoneurons projecting to muscles directly involved in copulation (TPN and RPM) as well as two other muscles (CF and SC; present data; Ruiz and Wade, 2002). More than half of this motoneuron population projects to the copulatory muscles (Table 1). Adult female anoles do not possess either TPN or RPM muscles, but like males, their T17-S1 nucleus projects to the CF and SC. Unilateral Injection of a retrograde tracer into the TPN, RPM, and CF all resulted in ipsilateral labeling of motoneurons. Injection of the SC, which wraps around the cloaca, results in bilateral labeling of motoneurons. For all muscles (TPN, RPM, CF, and SC), labeled motoneurons were located in the lateral ventral horn and were interdigitated throughout spinal segments T17-S1; specific sub-populations were not obviously segregated by medial-lateral or rostral-caudal position.

Organization of T17-S1 motoneuron projections

Retrograde tracer injections into the TPN, RPM, CF, and SC resulted in labeling of the vast majority of T17-S1 motoneurons in males (Table 1), suggesting that these four muscles comprise all major muscle targets for this motoneuron population. However, in adult females, while FB injections into the

CF and SC labeled approximately two-thirds of the T17-S1 motoneurons, not all were accounted for (Table 1). This discrepancy between males and females may be attributed to variability between the sexes in either the degree to which tracer filled the respective muscles or in transport efficacy. However, analysis of the injection sites revealed no obvious sex difference in the extent to which each muscle (CF and SC) was filled with FB. Also, a sex difference in transport efficacy may exist, but if so, it is likely due to a factor other than circulating testosterone levels; gonadal androgens do not affect axonal transport of another retrograde tracer, cholera toxin-conjugated horseradish peroxidase (Leslie et al., 1991).

Another possibility is that females have motoneurons in the T17-S1 nucleus that project to an additional muscle(s), as yet unidentified. Other leg and tail muscles are located near the CF and SC, including the retractor medialis, the retractor lateralis, and the protractor commissurae (Arnold, 1984). Perhaps the best candidate is the iliocaudalis tail muscle (ILC; also called the flexor caudae externus; Akita, 1992). This muscle is present in both sexes and lies adjacent to the CF (in both sexes) and TPN (in males), and the ILC and TPN are both supplied by the same nerve in male Iguanas (Akita, 1992). Additional projections in T17-S1 in females would be particularly interesting. As virtually all (96%) of these motoneurons have been identified in males with TPN, RPM, CF, and SC injections, it would mean that females have T17-S1 projections that are minimal or non-existent in males.

Alternatively, female anoles may have residual motoneurons in the T17-S1 nucleus that do not actually project to muscles. Female lizards do develop hemipenes and the corresponding musculature embryonically, however, in some species, including anoles, these structures regress by hatching (Dufaure and Hubert, 1961; Raynaud and Pieau, 1985; Holmes and Wade, in press; Chapter 5). It would be exciting if a subset of the corresponding motoneurons were maintained into adulthood without target musculature (perhaps via glial support), which may be consistent with previous reports in mammals (Glicksman et al., 1998). In at least one other reptile species, leopard geckos (*Eublepharis macularius*), adult females can develop fully evertable hemipenes when treated with testosterone (Rhen et al., 1999). The degree to which this hemipenis plasticity is accompanied by corresponding development of TPN and RPM musculature and motoneurons is currently under investigation. One possibility is that female leopard geckos maintain copulatory motoneurons into adulthood, which then support function of the remainder of the system when it develops (as opposed to having adult motoneuron genesis, which would be exciting but quite surprising).

Finally, given that both females and males have motoneurons projecting to the CF and SC, it appears that the sex difference in T17-S1 motoneuron number (present data; Ruiz and Wade, 2002) is largely due to females not having TPN or RPM motoneurons (but see above). One prediction that arises from this fact is that females should have higher *percentages* of cells projecting to CF and SC given their lower number of total T17-S1 motoneurons, and indeed they do,

although the effect is certainly more striking for SC (27% in females versus 9% in males) than CF (43% in females versus 32% in males) projections (Table 1).

Implications for structure/function relationships within this system

The ipsilateral projections to the TPN and RPM are consistent with the organization of copulatory behavior in this species. Male anoles use only one hemipenis per copulation (Crews, 1978), suggesting that each is independently controlled by its own neuromuscular system. Furthermore, over relatively short periods of time, male anoles alternate hemipenis use in order to maximize sperm transfer (Tokarz, 1988; Tokarz and Slowinski, 1990; Tokarz and Kirkpatrick, 1991); green anoles in particular rarely use individual hemipenes more than two times in succession (Crews, 1978). While there is evidence to suggest that sensory feedback from the hemipenes and/or testes mediates this effect in part (Crews, 1978), it may be that muscular fatigue also participates in this behavioral pattern.

The bilateral labeling of motoneurons observed following injection of the SC is consistent with at least two avian species (Japanese quail and Brown Leghorn fowl), in which, similar to the present data, motoneurons projecting to the SC are located in the pelvic region of the spinal cord (Ohmori and Watanabe, 1989; Seiwert and Adkins-Regan, 1998). This bilateral labeling may be because the SC receives bilateral projections with axons crossing the midline. Alternatively, the SC may receive only ipsilateral projections, but they could not

be detected because the muscle wraps around the cloaca and all injections filled fibers on both sides.

Finally, interdigitation of T17-S1 motoneurons projecting to different muscles is consistent with nerve organization in other reptilian and avian species. In male Iguanas, RPM, CF, and SC muscles all receive projections via the same nerve trunk, which branches into the pudendal nerve (serving SC and RPM) and the nerve serving the CF (Akita, 1992). Furthermore, SC and CF motoneurons are interdigitated and may share the connexus caudalis nerve in domestic fowl (Ohmori and Watanabe, 1989). This organization of the nerve is not surprising given the close proximity of these muscles within the lizard tail as well as the fact that, arguably, all four muscles are used (either directly or indirectly) in copulation. While the TPN and RPM are directly involved in the control of the hemipenis during intromission, the CF is likely involved in postural positioning during mounting and the SC may also be involved in facilitating intromission, either by passively or actively permitting the hemipenis to extend through the cloaca.

Comparison to organization of other dimorphic neuromuscular systems

In male anoles, more than half of T17-S1 motoneurons project to the TPN and RPM, both muscles critical for male copulatory behavior. The remaining motoneurons project to muscles that are not *directly* involved in copulation: the CF (a leg muscle) and SC. This composition is reminiscent of spinal nuclei in laboratory rats in which the dorsolateral nucleus and spinal nucleus of the

bulbocavernosus, both sexually dimorphic, have motoneurons projecting to both copulatory and non-copulatory muscles (e.g. Schroder, 1980; McKenna and Nadelhaft, 1986), including external sphincters involved in waste elimination. Interestingly, in the neuromuscular systems underlying vocalization in fish, frogs, and songbirds, while populations of motoneurons within brain nuclei can project to different muscle targets, those projecting to sexually dimorphic muscles are usually clustered, rather than interdigitated, populations (Nottebohm et al., 1976; Bass, 1985; Simpson et al., 1986).

Sex differences in neuromuscular systems are often not limited to motoneuron somata and muscle size. For example, they can include dimorphisms in dendritic arborization, neuromuscular junction size, neurotransmitter release, and synaptic efficacy (reviewed in Breedlove et al., 2002). Indeed, in the neuromuscular system that extends the anole dewlap during *courtship*, not only do adult males have larger motoneurons and muscle fibers, they also have longer cartilage, more muscle fibers, larger nerve cross sectional area, and larger neuromuscular junctions than adult females (O'Bryant and Wade, 1999; O'Bryant and Wade, 2002b). Now that organization of the anole *copulatory* neuromuscular system is better understood, we are primed to uncover the full extent of dimorphisms in this set of structures and the mechanisms regulating them. This work can then be related to that from copulatory systems of mammalian species. Importantly, it will also allow direct comparisons between copulatory neuromuscular systems and those regulating courtship, traditionally studied in non-mammalian vertebrates. In anoles, both

types of dimorphic neuromuscular systems are required for the full suite of masculine reproductive behaviors.

CHAPTER TWO

Holmes MM, Wade J (2004). Seasonal plasticity in the copulatory neuromuscular system of green anole lizards: a role for testosterone in muscle but not motoneuron morphology. J Neurobiol 60: 1-11.

INTRODUCTION

Sexually dimorphic neuromuscular systems exhibit sensitivity to androgens in adulthood in diverse vertebrate species. For example, vocal courtship behavior and morphology of the neural and muscular components underlying it are regulated by androgens in fish, frogs, and birds (*e.g.* Kelley and Pfaff, 1976; Hannigan and Kelley, 1986; Sassoon et al., 1987; Tobias et al., 1991; Brantley et al., 1993b; Brenowitz and Lent, 2002; Girgenrath and Marsh, 2003). Muscles and motoneurons in the copulatory system of laboratory rodents also enlarge in response to androgens (Breedlove and Arnold, 1981; Balice-Gordon et al., 1990; Rand and Breedlove, 1992, 1995) and, in parallel, are plastic in species exhibiting naturally occurring seasonal fluctuations in testosterone (T; Forger and Breedlove, 1987; Hegstrom and Breedlove, 1999; Hegstrom et al., 2002).

Green anoles (*Anolis carolinensis*) are lizards that breed seasonally, from about April to July, in the southeastern United States. Males court females by performing a suite of head bobbing displays coupled with the extension of a bright red throat fan, called a dewlap. Copulation occurs when a male flips his tail under a female's and inserts one of his two copulatory organs (called hemipenes) into her cloaca. T in males is about twice as high during the breeding than the non-breeding season (Lovern et al., 2001), and is important for the expression of reproductive behaviors; castration of males decreases their frequency and T replacement prevents or reverses this effect (Mason and Adkins, 1976; Crews et

al., 1978; Adkins and Schlesinger, 1979; Winkler and Wade, 1998; Rosen and Wade, 2000).

The dewlap and the neuromuscular system controlling its extension are sexually dimorphic. Male dewlaps are approximately 7-fold larger in extended area compared to those of females (Jenssen et al., 2000). Males extend their dewlaps more frequently than females (Nunez et al., 1997), even if females are given doses of T equivalent to males (Winkler and Wade, 1998). Only males use the dewlap in courtship, although both sexes will extend their dewlaps in aggressive encounters (Greenberg and Noble, 1944; Jenssen and Nunez, 1998; Nunez et al., 1997). Parallel to the behavioral dimorphism, the cartilage, motoneurons, nerve, neuromuscular junctions, and muscle fibers required for dewlap extension are all larger in males than females (Wade, 1998; O'Bryant and Wade, 1999; O'Bryant and Wade, 2002b). The morphology of this system does not appear to vary across season or respond to changes in T (O'Bryant and Wade, 1999).

Like the dewlap system, the copulatory neuromuscular system in green anoles is dimorphic on multiple levels (Ruiz and Wade, 2002). Male anoles possess bilateral hemipenes in the tail, which are controlled ipsilaterally by two sets of muscles. The transversus penis (TPN) wraps over the hemipenis and its contraction everts the hemipenis through the cloaca; the retractor penis magnus (RPM) is attached to the base of the hemipenis and retracts it back into the tail (Arnold, 1984). Adult females do not possess hemipenes or either of the two muscles (Ruiz and Wade, 2002). The motoneurons projecting to the TPN and

RPM are located in the pelvic region of the spinal cord in segments trunk 17 and sacral 1 (T17-S1; Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1), and these cells are smaller in size and number in females compared to males (Ruiz and Wade, 2002).

It was not known whether the copulatory neuromuscular system of male anoles is plastic in adulthood and which, if any, components of this system are sensitive to changes in environmental condition and/or androgens. Therefore, the goals of the present investigation were to determine whether, parallel to the changes in reproductive behavior, (Experiment 1) seasonal changes in the morphology of the copulatory system exist in gonadally intact male green anoles and (Experiment 2) whether such changes are mediated via gonadal androgens.

MATERIALS AND METHODS

Housing conditions and care

Adult male green anoles were purchased from Charles Sullivan Co (Nashville, TN) at three different times of year and were individually housed in 21-liter glass aquaria (42 x 20 x 24cm) for a minimum of 14 days (Experiment 1) or 7 days (Experiment 2). Lizards purchased during April (the beginning of the breeding season in the field) were housed under environmental conditions conducive to breeding. Animals were exposed to a 14:10 hour light/dark cycle using fluorescent, full-spectrum, and heat lamps. Temperature ranged from 28°C (ambient) to 38°C (directly under the heat lamp over each cage) during the day and was 18°C at night. These animals all had reproductive testes (see below)

and were classified as breeding season (BS) males. Lizards purchased in September (after the field breeding season) were housed in the same conditions but had regressed testes (BS-X). This combination of breeding environmental conditions but regressed testes does not occur naturally in this species, but allows independent analysis of environmental conditions and reproductive status. Finally, lizards purchased in November were housed in simulated non-breeding conditions including a 10:14 hour light/dark cycle, with temperature ranging from 24°C (ambient) to 30°C (directly under heat lamp) during the day and 15°C at night. These animals had regressed testes and were classified as non-breeding season (NBS) males. Thus we compared three experimental conditions: BS, BS-X, and NBS. In all cases, aquaria were sprayed daily to help maintain 70% relative humidity and water was provided ad libitum. Lizards were fed crickets or mealworms three times a week (BS and BS-X) or twice a week (NBS) as outlined in Lovern et al. (2004a).

Treatment and tissue collection

Experiment 1- Gonadally Intact Males. Adult males (n=8 per group) were overdosed with Brevital Sodium, weighed, measured snout to vent (SVL), and perfused with phosphate buffered saline (PBS) and 10% phosphate buffered formalin (PBF). Spinal column tissue was post-fixed in PBF for one hour, and segments were marked with ink to permit identification. The cords were then extracted (segments T15-S2), embedded in gelatin, post-fixed overnight in 20% sucrose in PBF, and sectioned frozen in the coronal plane at 30μm. Every other

section was mounted out of 9:1 dH2O:PBS onto gelatin-coated slides, stained with thionin, dehydrated and coverslipped using Permount.

The kidneys and rostral tail (containing the hemipenes, TPNs and RPMs) were post-fixed in Bouin's fixative for seven days. To provide better exposure of the tissues of interest to fixative and paraffin (see below), only the ventral half of the tail was maintained. The tissues were then soaked in 70% ethanol overnight, dehydrated, cleared in xylene, embedded in paraffin, and sectioned at 10μm. The tail tissue was stained with the trichrome method, and kidneys were stained with hematoxylin and eosin. In lizards, renal "sex segments" perform functions similar to the mammalian prostate and enlarge in response to androgen, thus they provide a bioassay for T exposure (e.g. Cueller et al., 1972; Crews, 1980; Winkler and Wade 1998).

Experiment 2 – Testosterone manipulation. Lizards were anesthetized using isoflurane and were bilaterally gonadectomized (GDX) while on ice. Condition of the testes (size, color, and vascularization) and vasa deferentia was noted. During the same surgery, each lizard was implanted subcutaneously with a Silastic implant (7mm long x 0.76mm ID x 1.65mm OD) containing either T proprionate (5mm packed) or left empty (n=8 per group) as in O'Bryant and Wade (1999). Twenty-one days following castration, lizards were overdosed with Brevital Sodium, body mass and SVL were noted, and they were perfused with PBS and PBF. Confirmation of the implant (including presence/absence of steroid) was done at the time of perfusion. Spinal cord, tail, and kidney tissue were all processed as described for Experiment 1.

Tissue Analyses

All measurements were conducted in the same manner for Experiments 1 and 2 without knowledge of experimental group or hormone manipulation (Experiment 2 only).

Motoneurons were analyzed as in Ruiz and Wade (2002). Briefly, the soma size of twenty randomly selected motoneurons was measured through the rostral-caudal extent of T17-S1 on each of the right and left sides using NIH Image. As a control, motoneuron soma size was measured for twenty motoneurons using the same technique in spinal segment T16; these motoneurons do not project to the TPN or RPM (Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1). The cross-sectional area of twenty-five randomly selected RPM fibers was measured per side (dispersed throughout the muscle). Caudifemoralis (CF) fiber size was measured using the same technique as a control; it is a leg muscle lying largely in the tail and is not specifically involved in copulation. Cross-sectional area of each hemipenis was determined in ten sections, approximately 50μm apart through the rostral ~500μm of the structures. The TPN was not analyzed as the fibers run perpendicular to those of the RPM and CF, so their cross-sectional area could not be determined in this tissue. Finally, the height of four renal sex segment epithelial cells was measured from each of 10 tubules randomly selected across the two kidneys (resulting in 40 measures; Winkler and Wade, 1998).

For all tissues except kidneys, a mean was calculated separately for each side in each individual. As the left and right sides did not differ in any case (data not shown), analyses reported below reflect an average of both sides for each structure (i.e., from a total of 40 motoneurons for both T17-S1 and T16, 50 muscle fibers for both RPM and CF, and 20 sections for hemipenes).

Statistical Analyses

Experiment 1. Each measure was analyzed by one-way ANOVA with experimental condition as the independent variable. Post hoc analyses were performed using Tukey-Kramer tests. One male was excluded from the analyses because, although his testes appeared reproductive (i.e., large, white and vascularized), his renal sex segment cell height was more than two standard deviations below the mean and his vasa deferentia were regressed at the time of perfusion, suggesting he was not responsive to circulating T. Thus, the final sample size in the BS group was 7, compared to n=8 in the BS-X and NBS groups.

Experiment 2. Each measure was analyzed by two-way ANOVA with experimental condition and androgen treatment as independent variables. Post hoc analyses, to test effects among the three conditions (BS, BS-X, and NBS), were performed using Tukey-Kramer tests; planned comparisons were performed to test effects of T within each experimental condition. Four males were excluded from analyses: one BS+GDX male because his renal sex segment cell height was more than two standard deviations above the mean,

suggesting that his T levels were not representative of a castrated male; one BS+GDX+T male because the implant fell out one week prior to perfusion; and two males died prior to perfusion (one NBS+GDX and one NBS+GDX+T). Thus, measurements were obtained from 7 or 8 animals per group. All analyses were performed using Statview (SAS Institute).

RESULTS

Experiment 1

Motoneuron soma size. T17-S1 motoneuron somata were smaller in NBS males than in BS or BS-X males ($F_{2, 20} = 3.64$; $p = 0.045$; Figure 1A), although Tukey-Kramer tests failed to detect statistically significant differences. In the control region (T16), the effect of experimental condition was not statistically significant. However, the same pattern was detected ($F_{2, 20} = 3.22$; $p = 0.061$; Figure 1B).

Peripheral copulatory structures and general measures. Experimental condition did not affect RPM ($F_{2, 20} = 0.40$; $p = 0.677$; Figure 1C) or CF fiber size ($F_{2, 20} = 2.42$; $p = 0.115$; Figure 1D), nor did it affect hemipenis cross sectional area ($F_{2, 19} = 0.80$; $p = 0.463$; Figure 1E). However, renal sex segment cell height was increased in BS compared to BS-X and NBS males ($F_{2, 20} = 148.95$; $p < 0.001$; both Tukey-Kramer $p < 0.05$; Table 1). No significant differences among groups were detected for body mass ($F_{2, 20} = 0.01$; $p = 0.994$) or SVL ($F_{2, 20} = 0.33$; $p = 0.724$).

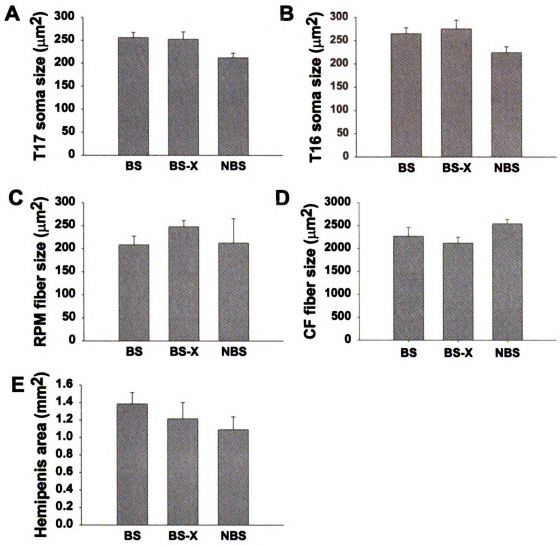

Figure 1. Effects of experimental condition on (A) T17-S1 motoneuron soma size, (B) T16 soma size, (C) RPM muscle fiber size, (D) CF fiber size, and (E) hemipenis cross sectional area in gondally intact males. In panel A, a main effect was detected without any significant pairwise comparisons (see Results).

	BS	BS-X	NBS
RSS height (µm)	31.30 (1.36)*	10.52 (0.83)	11.53 (0.57)
Body mass (g)	5.30 (0.29)	5.31 (0.21)	5.34 (0.25)
SVL (mm)	64.57 (0.65)	65.0 (0.93)	63.94 (1.14)

Table 1. Mean (+/- SEM) renal sex segment (RSS) cell height, body mass and snout-vent length (SVL) for gonadally intact in Experiment 1. * indicates statistically different from both other groups on same measure.

Experiment 2

Motoneuron soma size. As in Experiment 1, experimental condition affected T17-S1 motoneuron soma size ($F_{2, 38} = 8.87$; $p < 0.001$; Figure 2A). NBS and BS (both Tukey-Kramer $p < 0.05$) males had smaller somata than BS-X males. Androgen treatment did not affect T17-S1 soma size ($F_{1, 38} = 0.10$; $p = 0.757$), and the interaction between experimental condition and T-treatment was not statistically significant ($F_{2, 38} = 0.32$; $p = 0.730$). Experimental condition affected T16 motoneuron soma size ($F_{2, 38} = 10.09$; $p < 0.001$; Figure 2B), although androgen treatment did not ($F_{1, 38} = 1.83$; $p = 0.184$). As with T17-S1 motoneurons, NBS and BS (both Tukey-Kramer $p < 0.05$) males had smaller T16 somata than did BS-X males. Again, no significant interaction between condition and androgen was present ($F_{2, 38} = 0.82$; $p = 0.446$).

Muscle fiber size. RPM fiber size was unaffected by experimental condition ($F_{2, 37} = 1.07$; $p = 0.354$; Figure 2C), and this variable did not significantly interact with androgen treatment to alter RPM size ($F_{2, 37} = 0.98$; $p = 0.387$). However, T-treatment did significantly increase RPM fiber size ($F_{1, 37} = 14.52$; $p < 0.001$; Figure 3). Planned comparisons reveal that GDX+T males had larger RPM muscle fibers than GDX males in the BS ($p = 0.025$) and BS-X ($p = 0.002$) conditions but *not* in the NBS condition ($p = 0.387$). Both androgen treatment ($F_{1, 37} = 5.68$; $p = 0.022$) and experimental condition ($F_{2, 37} = 4.47$; $p = 0.018$) significantly affected CF fiber size (Figure 2D), but they did not interact ($F_{2, 37} = 0.05$; $p = 0.951$). BS males had smaller CF muscle fibers than BS-X males

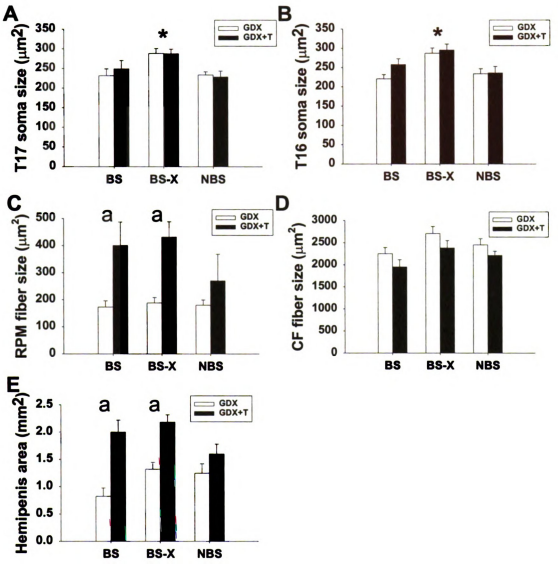

Figure 2. Effects of experimental condition and testosterone manipulation on (A) T17-S1 motoneuron soma size, (B) T16 soma size, (C) RPM muscle fiber size, (D) CF fiber size, and (E) hemipenis cross sectional area in castrated males (GDX = with blank implant; GDX+T = with testosterone implant). * indicates significantly different from other experimental groups (among BS, BS-X and NBS). [a] indicates a significant difference between GDX and GDX+T males within an experimental group. In panel D, a main effect of testosterone manipulation was detected without any significant pairwise comparisons; an effect of experimental condition stems from a significant difference between the BS and BS-X groups only (see Results).

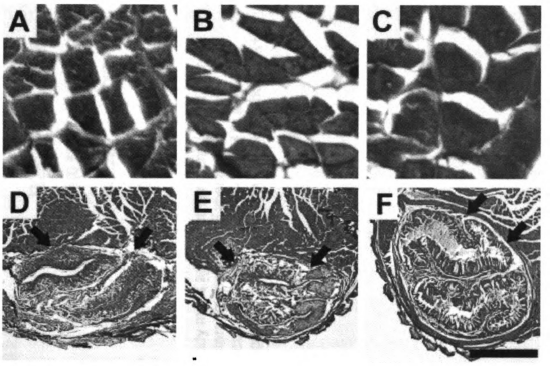

Figure 3. Photomicrographs of cross-sections of RPM muscle fibers (A, B, C) and hemipenes (D, E, F) taken from BS males. A and D are from a gonadally intact male, B and E are from castrated males with blank capsules, and C and F are from a castrated male with a testosterone implant. Arrows point to the dorsal edge of each hemipenis, where it meets the caudifemoralis muscle. Scale bar = 25μm (A, B, C) and 800μm (D, E, F).

	BS		BS-X		NBS	
	GDX	GDX+T	GDX	GDX+T	GDX	GDX+T
RSS height (µm)	15.13(1.4)	40.94(0.91)[a]	10.51(0.76)	35.96(1.66)[a]	10.96(0.55)	27.05(1.69)[a]
Body mass (g)	4.76(0.18)*	4.52(0.26)*	5.27(0.21)	5.13 (0.30)	5.39(0.22)	4.57(0.17)
SVL (mm)	60.86(0.60)	60.71(1.04)	65.50(0.87)**	65.88(0.61)**	65.00(1.33)	61.00(1.77)

Table 2. Mean (+/- SEM) renal sex segment (RSS) cell height, body mass and snout-vent length (SVL) for castrated males (GDX = with blank implant; GDX+T = with testosterone implant) in Experiment 2. For comparisons across experimental conditions, * indicates a significant difference from BS-X, and ** indicates significant differences from both other conditions. [a] indicates a significant difference between GDX and GDX+T groups within same experimental condition.

(Tukey-Kramer $p < 0.05$) but not NBS males ($p > 0.05$). GDX+T males overall had slightly, but significantly, *smaller* CF muscle fibers than controls. Despite this main effect of androgen treatment, no significant effects of T on CF fiber size were detected within any of the 3 conditions with planned comparisons (all $p > 0.183$).

Hemipenis cross-sectional area. A trend for breeding season conditions to increase hemipenis size was detected ($F_{2, 38} = 2.86$; $p = 0.070$; Figure 2E), as was a trend for an interaction between experimental condition and androgen ($F_{2, 38} = 3.02$; $p = 0.061$). While overall GDX+T males had larger hemipenes than GDX controls ($F_{1, 38} = 35.18$; $p < 0.001$; Figure 3), planned comparisons demonstrate that this is true for the BS ($p < 0.001$) and BS-X ($p < 0.001$) groups, but not the NBS group ($p = 0.179$).

General measures. Androgen treatment ($F_{1, 37} = 480.17$; $p < 0.001$) and breeding environmental conditions ($F_{2, 37} = 24.53$; $p < 0.001$) increased renal sex segment cell height (Table 2). A significant interaction between experimental condition and androgen treatment was also detected ($F_{2, 37} = 9.58$; $p < 0.001$) with T-treatment increasing cell height more in breeding (BS and BS-X) than non-breeding (NBS) conditions. Despite random selection of individuals, those in the BS-X condition had significantly increased SVL ($F_{2, 38} = 10.43$; $p < 0.001$) compared to either BS or NBS (both Tukey-Kramer $p < 0.05$) males. T treatment did not affect SVL ($F_{1, 38} = 1.99$; $p = 0.166$), and no statistically significant interaction was detected ($F_{2,}$

$_{38}$ = 2.38; p = 0.106; Table 2). T-treatment ($F_{1, 38}$ = 4.50; p = 0.041) and breeding conditions (trend: $F_{2, 38}$ = 3.10; p = 0.057) both increased body mass without a significant interaction ($F_{2, 38}$ = 1.25; p = 0.299; Table 2). BS-X males were heavier than BS (Tukey-Kramer p < 0.05) but not NBS males. These group differences in body mass were also present prior to surgery (data not shown) demonstrating that they are not due to the treatment.

DISCUSSION

Summary

The results from Experiment 1 demonstrate that environmental condition can alter soma size in sexually dimorphic spinal motoneurons (T17-S1); males housed in non-breeding conditions had smaller somata than did males housed in breeding conditions. A trend for the control motoneurons (T16) to be smaller in this group also existed, suggesting that the effect of non-breeding conditions on soma size may not be specific to copulatory motoneurons. No seasonal variation was detected in any other measure in gonadally intact animals, with the exception of renal sex segment cell height, confirming that circulating androgen levels vary across season (Lovern et al., 2001). Results from Experiment 2 reveal that neither T17-S1 nor T16 motoneurons are sensitive to changes in circulating T in adults. However, while *both* hemipenis and RPM fiber size increased in response to T, this effect was only detected in males housed under conditions simulating the breeding season. In contrast, T slightly, but significantly, decreased CF muscle fiber size across the three experimental

conditions, indicating that the trophic effect of T on copulatory structures was relatively specific. Taken together, the data demonstrate that adult morphological plasticity is regulated by different mechanisms in copulatory motoneurons compared to the hemipenes and RPMs. While modest changes in motoneuron soma size might occur seasonally on a general level, environmental cues mediate T-induced changes in peripheral copulatory structures.

Plasticity in motoneuron soma size

The decrease in copulatory motoneuron soma size during the non-breeding season is consistent with reports in mammals (Forger and Breedlove, 1987; Hegstrom et al., 2002). However, in contrast to the two mammalian species studied to date (the white-footed deer mouse, *Peromyscus leucopus*, and the Siberian hamster, *Phodopus sungorus*), seasonal plasticity in the soma size of anole copulatory motoneurons appears independent of T. That is, while circulating T in the green anole is approximately twice as high during the breeding compared to the non-breeding season (Lovern et al., 2001), T-treatment did not increase T17-S1 soma size in castrated males housed in either condition. As season may also affect non-copulatory motoneurons (T16), the somewhat smaller soma size in the non-breeding season is likely a relatively general effect of variations in environmental condition. Indeed, physical activity, which is higher during the breeding season in the anole (Jenssen et al., 1995; Jenssen et al., 1996), mediates motoneuron soma size; decreased activity can

be associated with smaller somata in some neural populations (e.g. Nakano et al., 1997; Nakano and Katsuta, 2000; but see Breedlove, 1997a).

In Experiment 2, both BS and NBS males had smaller motoneurons than BS-X males. While the decrease in NBS soma size may relate to seasonal factors (as in Experiment 1), the difference between BS and BS-X males is likely due to smaller SVL and body weight (Table 2). These differences, not seen in Experiment 1, are an unfortunate artifact of random assignment of males to experimental groups. Indeed, if T17-S1 soma size is corrected for body weight or SVL, then the significant difference between BS and BS-X groups is no longer present (soma size/body weight, Tukey-Kramer $p > 0.05$; soma size/SVL, Tukey-Kramer $p > 0.05$), whereas the difference between BS-X and NBS groups is maintained (soma size/body weight, Tukey-Kramer $p < 0.05$; soma size/SVL, Tukey-Kramer $p < 0.05$). In any case, the point of interest in the motoneuron data from Experiment 2 is that androgen manipulation failed to alter soma size regardless of environmental condition, suggesting that, in contrast to mammalian species (both seasonal breeders and laboratory rats), the soma size of copulatory motoneurons is relatively insensitive to androgen in adult anoles. However, other structural (e.g., dendritic morphology; Rand and Breedlove, 1995) or functional (e.g., neurotransmitter release; Tobias and Kelley, 1995) properties that were not evaluated in the present experiment, may be altered by T.

Plasticity in androgen sensitivity in hemipenes and RPM muscle fibers

While the size of peripheral copulatory structures does not change seasonally in gonadally intact male anoles, the RPMs and hemipenes exhibit seasonal plasticity in their sensitivity to androgen manipulation. That is, T-treatment dramatically increased hemipenis and RPM fiber size, but only when animals were housed in environmental conditions conducive to breeding (long days and warm temperatures). One possible explanation for the seasonal variation in responsiveness to T is that it serves to protect the copulatory system from morphological regression during periods of lower circulating androgens. During the breeding season (BS), the reduction in peripheral structures in GDX compared to intact males is consistent with this idea (compare Figures 1C and E to Figures 2C and E). However, unmanipulated males still have approximately 10ng/ml of plasma T during the non-breeding season (compared to ~20ng/ml during the breeding season; Lovern et al., 2001), which may be sufficient to maintain the morphology (consistent with results from the gonadally intact males in Experiment 1). Alternatively, the increased dynamic range under breeding environmental conditions (BS and BS-X) may facilitate the response of the system to even relatively small T fluctuations that may occur with particular experiences (e.g., exposure to females; reviewed in Hart, 1983).

The dose of T used in the present experiments is likely supraphysiological given that renal sex segment cell height is larger in males treated with T than gonadally intact breeding males (compare Tables 1 and 2). While physiological variations in T may not be substantial enough to induce morphological changes in the RPMs and hemipenes of gonadally intact males, the *potential* for

substantial plasticity clearly exists in these structures. And, perhaps more interesting is the manner in which this potential for response to T varies across environmental conditions. This pattern is similar to alterations in sensitivity to T in the rodent brain. Neuron soma size in the medial amygdala of Siberian hamsters increases in response to T-treatment only when animals are housed in long photoperiods (Cooke et al., 2002). The mechanism for the changes in sensitivity is presently unknown but may be mediated by differences in androgen receptors; their distribution in the copulatory structures of anoles must be investigated.

Similar to seasonal changes in sensitivity to T, lizards show reduced responsiveness to adrenocorticotropin hormone (Carsia and John-Alder, 2003) and melatonin (Bertolucci and Foa, 1998) during the non-breeding season. Indeed, melatonin is essential for normal circadian and seasonal rhythmicity in lizards (e.g., Bertolucci and Foa, 1998; Bertolucci et al., 2002; Foa et al., 2002), including green anoles (Hyde and Underwood, 1995; Hyde and Underwood, 2000), and may mediate activity of other hormones or independently create morphological change. For example, in the Siberian hamster, increased melatonin release facilitates gonadal regression and decreased androgen production (Kelly et al., 1994). This pathway appears to induce shrinkage of copulatory motoneuron soma and muscle fiber size (Hegstrom and Breedlove, 1999; Hegstrom et al., 2002). However, photoperiod decreases neuromuscular junction size independent of T levels, consistent with a more direct role for melatonin (Hegstrom et al., 2002).

Different mechanisms in the copulatory vs. courtship neuromuscular systems

In adulthood, the sexually dimorphic neuromuscular system controlling dewlap extension exhibits little or no plasticity in response to season or androgen manipulation (O'Bryant and Wade, 1999), at first glance suggesting that morphological changes in this system do not parallel seasonal changes in behavior. However, while the frequency of extension is greatly reduced, both males and females do use their dewlaps in the non-breeding season occasionally (mostly for aggressive interactions; Jenssen et al., 1996). It is possible that this limited use requires or facilitates maintenance of the structures. The seasonal changes in copulatory motoneurons and sensitivity to androgen in peripheral copulatory structures also relates to behavior; male anoles *do not* copulate under short days and lower temperatures even when treated with T (O'Bryant and Wade, 2002a).

Comparisons to other species

Neuromuscular systems involved in male reproductive behaviors are sexually dimorphic in diverse vertebrate species, and gonadal steroids often mediate the maintenance of these dimorphisms (*e.g.* Kelley, 1986; Brantley et al., 1993b; Wade, 1999; Breedlove et al., 2002). For example, adult T-manipulation modulates morphology of the neuromuscular portion of the avian song system (*e.g.* Arnold, 1980; DeVoogd et al., 1991; Wade and Buhlman, 2000). Similarly, the mass of external oblique muscles of gray tree frogs (used in sound production) is larger in breeding males than in females and non-breeding

males and is increased by T (Girgenrath and Marsh, 2003). Auditory sensitivity in female midshipman fish changes seasonally to match differences in male sound production (Sisneros and Bass, 2003), and may be due to gonadal steroids (Forlano et al., 2003). It is unknown, however, whether the structures underlying vocal production exhibit seasonal morphological plasticity. Finally, photoperiod and androgens enhance the copulatory neuromuscular system of seasonally breeding mammalian species (Forger and Breedlove, 1987; Hegstrom et al., 2002). Collectively these data demonstrate that sex and seasonal differences in morphology often parallel function and, furthermore, that T often mediates morphological changes in adulthood. Likewise, components of the anole copulatory system and the behaviors they facilitate are highly sensitive to T, but the responsiveness depends on environmental cues. It will be important to determine the specific mechanisms underlying this effect and whether they are involved in other sexually dimorphic vertebrate systems.

CHAPTER THREE

Holmes MM, Wade J. Testosterone regulates androgen receptor immunoreactivity in the copulatory, but not courtship, neuromuscular system in adult male green anoles. J Neuroendo: submitted.

INTRODUCTION

Androgens commonly regulate the expression of masculine reproductive behaviors in a wide variety of vertebrate groups, including fish (Brantley et al., 1993c), frogs (Wetzel and Kelley, 1983), birds (Arnold, 1975; Harding et al., 1983), and mammals (Hart, 1967). While the motivation for such behaviors is mediated by hypothalamic and preoptic brain regions (Hull et al., 2002), the execution of courtship and copulatory behaviors requires neuromuscular systems, which are frequently sexually dimorphic (Breedlove et al., 2002). Androgens serve not only to activate the behaviors in adulthood, but also often mediate the development and maintenance of the underlying neuromuscular components (Breedlove et al., 2002). These effects on motoneuron and muscle morphology in particular usually occur by direct effects of androgenic steroids on the androgen receptor (AR) (Breedlove et al., 2002). As such, determining the distribution and regulation of AR is critical for understanding mechanisms that regulate structural and functional change in reproductive neural circuits.

Green anoles (*Anolis carolinensis*) are a particularly useful model species with which to study steroid hormone effects on structure and function because they possess two sexually dimorphic neuromuscular systems. One is involved in courtship behavior, which includes extension of a red throat fan called a dewlap. While both sexes possess dewlaps, they are approximately seven-fold larger in males than females (Jenssen et al., 2000), and males extend their dewlaps far more frequently than females (Greenberg and Noble, 1944; Nunez et al., 1997; Jenssen and Nunez, 1998). Paralleling the sex difference in size and overall use of the

dewlap, the neuromuscular components that mediate its extension are larger in males than females. Specifically, extension of the dewlap is caused by contraction of the bilateral ceratohyoideus (CH) muscles, which lie on the ventral surface of the throat and between the first ceratobranchial and ceratohyal cartilages (see schematic in Wade, 2005). Contraction of the CH causes the cartilages to act as a lever, ultimately bowing out the second ceratobranchial cartilages, which are found at the midline just under the skin on the ventral surface of the throat (Bels, 1990; Font and Rome, 1990). CH muscle fibers are larger in size and number and the second ceratobranchial cartilages are longer in males than females (O'Bryant and Wade, 1999; Lovern et al., 2004b; Chapter 4). In addition, the CH muscles receive projections from two pools of motoneurons in the caudal brainstem: the vagal portion of the nucleus ambiguus (AmbX) and the glossopharyngeal region of nucleus ambiguus and the ventral motor nucleus of the facial nerve (AmbIX/VIImv) (Font, 1991; Wade, 1998). These motoneurons are larger in size, but not number, in males than females (O'Bryant and Wade, 1999).

The other sexually dimorphic neuromuscular system is involved in male copulatory behavior. Male anoles possess two intromittant organs called hemipenes, which lie inside the ventral surface of the tail, immediately caudal to the cloaca. Each hemipenis is controlled primarily by two muscles: the transversus penis (which causes eversion of the organ), and the retractor penis magnus (RPM; which retracts the hemipenis back into the tail; Arnold, 1984). After hatching, only males possess hemipenes and the associated muscles (Ruiz and Wade, 2002; Lovern et al., 2004b; Chapter 4; Holmes and Wade, in press; Chapter 5). Contraction of the muscles

occurs via the innervation of ipsilateral motoneurons located in spinal segments trunk 17 and sacral 1 (T17-S1). The motoneurons in this region of the spinal cord are a mixed pool, projecting to both copulatory (in males only) and non-copulatory (caudifemoralis and cloacal sphincter in both males and females) muscles (Holmes and Wade, 2004a; Chapter 1). The cells in this population are greater in both size and number in males than females (Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1).

As in other species, androgens are important for the expression of both courtship and copulatory behaviors in male green anoles. These lizards breed seasonally, from approximately April to July in the Southeastern United States and testosterone levels are about twice as high in the breeding than non-breeding season (Jenssen et al., 1995; Jenssen et al., 1996; Lovern et al., 2001). Testosterone is the most potent hormone for activating male courtship and copulatory behaviors. Castration reduces their expression, while treatment with testosterone prevents or reverses these effects (Mason and Adkins, 1976; Crews et al., 1978; Adkins and Schlesinger, 1979; Winkler and Wade, 1998; Rosen and Wade, 2000). As dihydrotestosterone also contributes to the expression of male reproductive behaviors (Crews et al., 1978; Adkins and Schlesinger, 1979; Rosen and Wade, 2000), while estrogens have little or no effect (Mason and Adkins, 1976; Crews et al., 1978; Winkler and Wade, 1998), a direct role for AR is likely.

Testosterone can also affect morphology in tissues important for reproduction, but it does so selectively. The hormone increases RPM fiber size and hemipenis cross-sectional area in males housed in breeding, but not non-breeding,

environmental conditions (Holmes and Wade, 2004b; Chapter 2). In contrast, neither season nor testosterone appears to alter CH fiber size (O'Bryant and Wade, 1999). Of particular interest is that testosterone does not affect motoneuron soma size in either the courtship or copulatory systems (O'Bryant and Wade, 1999; Holmes and Wade, 2004b; Chapter 2), which may relate to the fact that there is limited expression of AR protein and mRNA in the courtship motoneurons (Rosen et al., 2002). To help determine whether the differential effects of testosterone on the dewlap and copulatory neuromuscular structures are mediated by AR, we had two experimental goals. The first was to identify which of the other courtship and copulatory tissues, including the targets of both systems (cartilages and hemipenes), express AR. The second was to determine whether AR immunoreactivity changes in response to season or testosterone manipulation. We evaluated AR immunoreactivity in adult males housed in either breeding or non-breeding conditions that were either gonadally intact (Experiment 1) or castrated and treated with testosterone or vehicle (Experiment 2).

MATERIALS AND METHODS

Housing conditions and care

Wild-caught adult male green anoles were purchased from Charles Sullivan Co. (Nashville, TN) at two different times of year and were individually housed in 21-liter glass aquaria (42 x 20 x 24cm) for approximately 3 weeks prior to tissue collection (Experiment 1) or treatment (Experiment 2; see below). Lizards purchased during May (during the breeding season in the field) were

housed under environmental conditions conducive to breeding. Animals were exposed to a 14:10 hour light/dark cycle using fluorescent, full-spectrum, and heat lamps. Temperature ranged from 28°C (ambient) to 38°C (directly under a heat lamp over each cage) during the day and was 18°C at night. These animals all had reproductive testes (large and vascularized, see below) and were classified as breeding season males. Lizards purchased in October were housed in simulated non-breeding conditions including a 10:14 hour light/dark cycle, with temperature ranging from 24°C (ambient) to 30°C (directly under heat lamp) during the day and 15°C at night. These animals had regressed testes (darker, small, not vascularized) and were classified as non-breeding season males. For both breeding and non-breeding season animals, aquaria were sprayed daily to help maintain 70% relative humidity and food and water was provided as outlined in (Lovern et al., 2004a).

Treatment and tissue collection

Experiment 1- Gonadally Intact Males. Adult males (n=8 per group) were rapidly decapitated and spinal columns, tails, throats, and kidneys were extracted, flash frozen in isopentane chilled on dry ice, and stored at –80 °C. Condition of the testes (size, color, and vascularization) and vasa deferentia was noted. All tissues were frozen sectioned in cross-section in 6 series at 20µm, thaw mounted onto Superfrost Plus glass slides (Fisher Scientific) and stored at –80 °C. A single series of each spinal column, tail, and throat was processed for AR immunohistochemistry similar to (Rosen et al., 2002). Briefly, slides were

warmed to room temperature and fixed for 5 min in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS). Following a 30 minute incubation in 0.5% H_2O_2 to remove endogenous peroxidase and a 2 hour incubation in 10% normal donkey serum in 0.1M PBS with 0.2% Triton X-100, all tissue was incubated for 36 hours in PG21 rabbit polyclonal antibody (1.5 ug/ml for spinal cord tissue and 1.75 ug/ml for throat and tail tissue; Upstate) in 0.1M PBS with 0.2% Triton X-100 at 4°C. A biotinylated donkey anti-rabbit secondary antibody (1:500 for 90 minutes; Jackson Laboratories), Elite ABC peroxidase reagents (Vector Laboratories), and nickel-enhanced diaminobenzidine were used to visualize AR. Slides were rinsed with 0.1M PBS between all steps. Spinal column tissue was dehydrated and coverslipped with Permount and muscle tissue (throats and tails) was stained for 5 minutes with 4',6-Diamidino-2-Phenylindole (DAPI;1ug/ml; Sigma) to visualize nuclei, dehydrated and coverslipped with DPX mounting medium (Fluka). Following quantification of AR immunoreactive nuclei (see below), slides containing spinal cords were placed in xylene overnight to remove coverslips, and stained with thionin to permit visualization of all motoneurons. For muscle tissue, adjacent series were stained with hematoxylin and eosin for confirmation of DAPI estimates for number of AR immunoreactive nuclei (see below).

Kidneys were stained with hematoxylin and eosin. In lizards, renal "sex segments" perform functions similar to the mammalian prostate and enlarge in response to androgen, thus they provide a bioassay for relative levels of

androgen exposure (e.g. Winkler and Wade, 1998; Cueller et al., 1972; Crews, 1980).

Experiment 2 – Testosterone manipulation. Lizards were anesthetized using isoflurane and were bilaterally gonadectomized (GDX) while on ice. Condition of the testes and vasa deferentia was noted. During the same surgery, each lizard was implanted subcutaneously with a Silastic implant (7mm long x 0.76mm ID x 1.65mm OD) containing either testosterone proprionate (5mm packed) or left empty (n=8 per group) as in (O'Bryant and Wade, 1999; Holmes and Wade, 2004b; Chapter 2). Twenty-one days following castration, lizards were rapidly decapitated and tissues were collected as above. Confirmation of the implant (including presence/absence of steroid) and completeness of castration was also noted at this time. Spinal column, tail, throat, and kidney tissue were all processed as described for Experiment 1.

Tissue Analyses

All measurements were conducted in the same manner for Experiments 1 and 2 without knowledge of experimental group.

To estimate the percentage of AR+ nuclei in the CH and RPM muscles, the number of AR+ and AR- nuclei were determined in 30 randomly selected muscle fibers (per animal). First, photomicrographs of muscle fibers were taken using fluorescent illumination to visualize DAPI labeled nuclei. The same fibers were then photographed using transmitted light to visualize AR+ nuclei. Comparison of these images (demonstrated in Figure 1) permitted the total

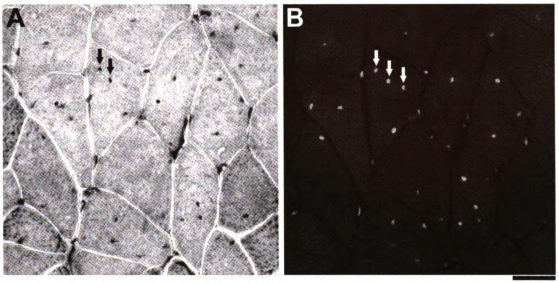

Figure 1. Photomicrograph of a cross-section through CH muscle fibers from a gonadally intact male housed in non-breeding environmental conditions illustrating expression of AR+ nuclei (A). (B) is the same section under fluorescent illumination to allow visualization of the DAPI nuclear label. Comparison of the number of nuclei in (A) and (B) provides an estimate for the total number of AR+ nuclei. Black arrows point to examples of AR+ nuclei and white arrows point to DAPI labeled nuclei. Scale bar = 30μm.

number of nuclei and the total number of AR+ nuclei to be determined. Dividing total number of AR+ nuclei by the total number of DAPI labeled nuclei provided an estimate of the percentage that was AR+. In reptilian muscles, nuclei are located both near the center and on the edge of the fibers (see Figure 1). We only included centralized nuclei in these analyses to be confident that we were evaluating myonuclei (Monks et al., 2004); the luminescence from the fluorescent DAPI labeling extends slightly beyond the nuclei, and the edge of fibers cannot be confidently determined because they are so dark. Hematoxylin and eosin was then used to confirm the DAPI counts and more accurately identify nuclei that were close to the edge of a given fiber as well as those in other cell types between the fibers. In this case, the number of muscle fibers as well as the total number of hematoxylin stained and AR+ nuclei were estimated using Stereoinvestigator (Microbrightfield, Inc) in a single cross-section for each muscle with approximately 15 sampling areas per section. Nuclei were divided into three categories: centralized (myonuclei), edge (possibly myonuclei, satellite cells or fibroblasts), or those clearly outside the muscle fibers (most likely in fibroblasts). Dividing the number of AR+ nuclei by the total number of hematoxylin-stained nuclei provided estimates for the percentage of AR+ nuclei for each category. AR immunoreactivity was not evaluated in transversus penis muscle fibers because they run perpendicular to those of the RPM and therefore could not be viewed in cross-section in this tissue.

To estimate the percentage of AR+ T17-S1 motoneurons, the number of motoneurons with AR+ nuclei was counted. Following staining with thionin (see

above), the total number of motoneurons was estimated using the physical dissector technique (Gunderson, 1986; Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1) where a cell is counted if its nucleus comes into focus and disappears within a given section. Estimates of AR+ motoneurons and total cell number were obtained for both sides of the spinal cords (through the rostro-caudal extent of T17-S1) and averaged. As with muscle nuclei, dividing the total number of AR+ motoneurons with the total number of motoneurons provided an estimate of the percentage of AR+ cells. We did not measure AR expression in the courtship motoneurons because they have previously been shown to express little or no AR (Rosen et al., 2002) and do not respond morphologically to androgen or environmental (season) manipulations (O'Bryant and Wade, 1999).

The neuromuscular target structures were also evaluated for AR immunoreactivity. Presence or absence of detectable immunoreactivity was evaluated for the three bilateral pairs of cartilage that control dewlap extension: the first ceratobranchials, the second ceratobranchials, and the ceratohyals and in each of three components of the hemipenes: the lobe epithelium, the body of the lobe, and the clavulae (which provide structural support for the lobes during intromission).

Finally, the height of four renal sex segment epithelial cells was measured from each of 10 tubules randomly selected across the two kidneys (resulting in 40 measures; Winkler and Wade, 1998) to evaluate relative levels of circulating androgens.

Statistical Analyses

For Experiment 1, percent AR+ myonuclei from the DAPI analyses, percent AR+ motoneurons, and total number of muscle fibers were each analyzed in intact animals (between breeding and non-breeding males) with independent t-tests. Estimates of percent AR+ nuclei using hematoxylin and eosin was analyzed with a one-way repeated-measures ANOVA with season as the between subjects variable and nucleus type (centralized, edge, or outside of muscle fiber) as a within-subjects measure. For Experiment 2, percent AR+ myonuclei from the DAPI analyses, percent AR+ motoneurons, and total number of muscle fibers were analyzed by separate two-way ANOVAs with season and androgen treatment as independent variables. Estimates of percent AR+ nuclei in the muscles using hematoxylin and eosin were analyzed using two-way repeated-measures ANOVAs with season and androgen-treatment as between subjects variables and nucleus type (centralized, edge, or outside of muscle fiber) as a within-subjects measure. Post hoc analyses were performed using Tukey-Kramer tests. All analyses were performed using Statview (SAS Institute).

RESULTS

Experiment 1: Gonadally intact males

The percent of AR+ nuclei in the RPM muscle did not differ between breeding and non-breeding males based in either the DAPI ($t = 0.43$; $p = 0.68$; Figure 2) or hematoxylin and eosin ($F = 0.24$; $p = 0.63$; Table 1) analyses.

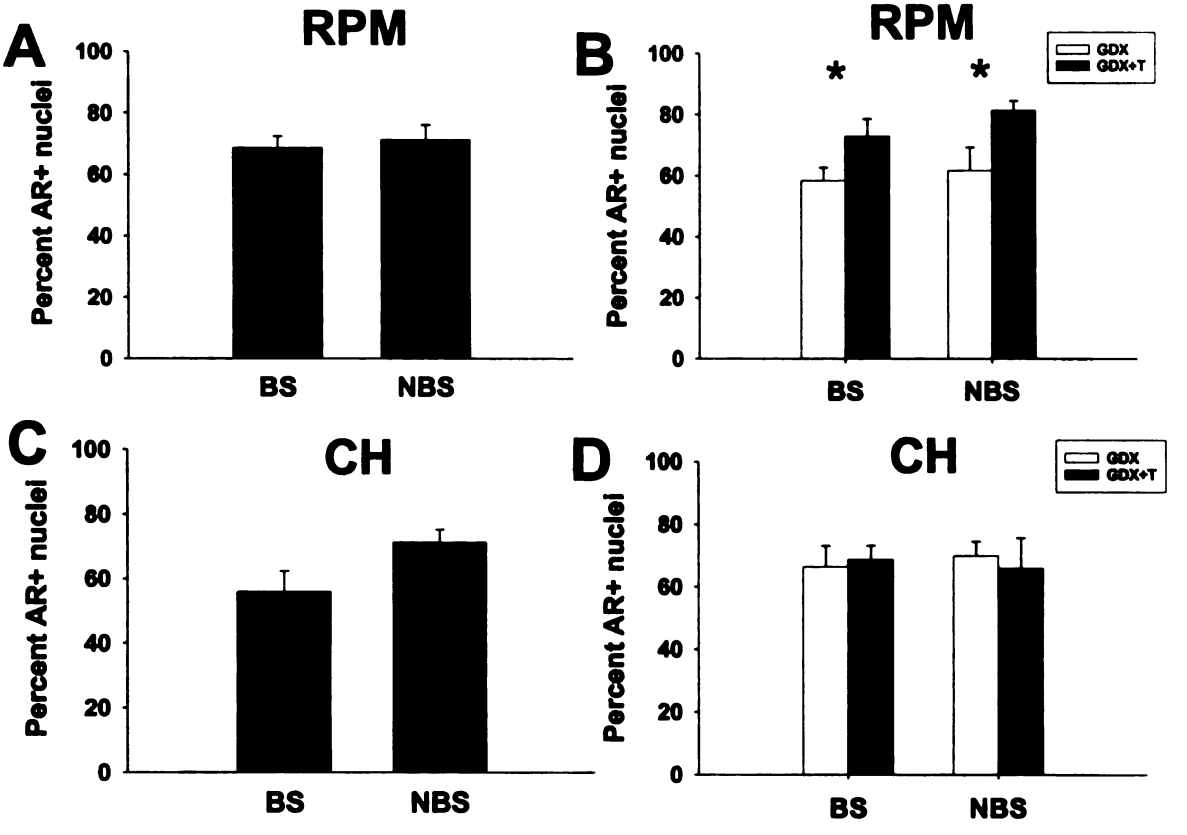

Figure 2. Mean (+/- SEM) percent of AR+ nuclei in the RPM (A and B) and CH (C and D). These values were obtained from DAPI labeled tissue. Panels (A) and (C) are from gonadally intact males (grey bars) and panels (B) and (D) are from castrated males (GDX) treated with testosterone (T; black bars) or empty capsules (white bars). Animals were housed either in breeding (BS) or non-breeding (NBS) environmental conditions. Asterisks indicate a statistically significant main effect of testosterone treatment on the percentage of AR+ nuclei.

	CH			RPM	
	Center	Edge	Outside	Center	Edge	Outside
Experiment 1						
BS	78.5 (9.0)	91.3 (13.4)	65.9 (9.0)	74.0 (14.1)	79.3 (12.2)	46.5 (10.8)
NBS	63.9 (10.5)	62.5 (9.0)	59.4 (9.9)	90.1 (18.2)	78.0 (18.7)	54.0 (9.6)
Experiment 2						
BS-GDX	96.6 (17.0)	80.9 (11.9)	71.6 (10.2)	67.7 (10.1)	65.8 (9.5)	53.9 (8.4)
BS-GDX+T	75.3 (8.1)	76.3 (7.5)	59.9 (5.2)	88.4 (9.2)*	96.2 (11.7)*	57.3 (6.2)*
NBS-GDX	79.3 (6.6)	79.4 (9.0)	65.2 (14.7)	58.6 (10.4)	55.8 (11.3)	37.7 (9.3)
NBS-GDX+T	74.6 (10.2)	77.3 (10.8)	54.8 (4.6)	116.2(16.4)*	104.1(13.3)*	83.5 (13.5)*

Table 1. Mean (+/-SEM) percent of AR+ nuclei from hematoxylin and eosin stained tissue. Nuclei were classified as in the center, on the edge, or outside of the muscle fibers for both the CH and RPM muscles. Asterisks indicate a significant main effect of testosterone (T) in castrated males (GDX) compared to GDX males treated with vehicle.

Interestingly, AR immunoreactivity significantly differed among different types of nuclei ($F = 4.70$; $p = 0.02$; Table 1), where the percentage was higher in centralized and edge nuclei compared to those completely outside of the muscle fibers (both $p < 0.05$), although no significant interaction was detected between season and nucleus type ($F = 0.29$; $p = 0.75$). The number of RPM muscle fibers did not differ between breeding and non-breeding males (331-404 fibers on average; $t = 1.00$; $p = 0.33$). AR immunoreactivity in T17-S1 motoneurons also did not differ between breeding and non-breeding males (on average, 75.32% for breeding males and 75.98% for non-breeding males; $t = 0.23$; $p = 0.82$; Figure 3), and while AR immunoreactivity was present in all three components of the hemipenes (Figure 4), the intensity of staining did not obviously differ across season.

Similar to the RPM, AR expression in the CH muscle did not significantly differ between breeding and non-breeding males in either the DAPI ($t=2.11$; $p = 0.06$; Figure 2) or hematoxylin and eosin ($F = 2.63$; $p = 0.13$; Table 1) analyses, and the interaction between nucleus type and season was not statistically significant ($F = 0.77$; $p = 0.47$). However, unlike the RPM, AR immunoreactivity was equivalent across nucleus type (centralized, edge, and outside of fibers) ($F = 1.25$; $p = 0.30$; Table 1). CH muscle fiber number did not differ between breeding and non-breeding males (295-300 fibers on average; $t = 0.22$; $p = 0.83$). AR labeling was distinct in the nuclei of both the first and second ceratobranchial cartilages, but was very faint in the ceratohyals (Figure 5) and did not obviously change across season in any case.

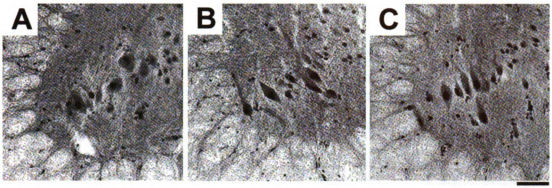

Figure 3. Photomicrographs of cross-sections through the ventral horn in spinal segment T17 from a gonadally intact male (A), castrated male (B), and castrated male treated with testosterone (C), all housed in breeding environmental conditions. The majority of motoneurons have nuclei that exhibit dark AR immunoreactivity; the percentage of AR+ cells not differ between experimental groups. Scale bar = 50μm.

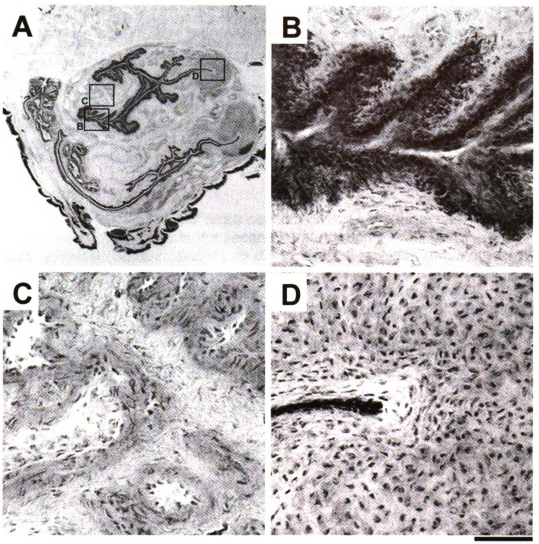

Figure 4. Photomicrographs of cross-sections through one hemipenis of a gonadally intact male housed in breeding conditions. Panel (A) is a low magnification photo of the entire cross-sectional area of the hemipenis. Panels (B-D) are high magnification photos of the lobe epithelium (B), lobe body (C), and clavula (D) from the hemipenis in (A). Scale bar = 600μm for (A) and 50μm for (B-D).

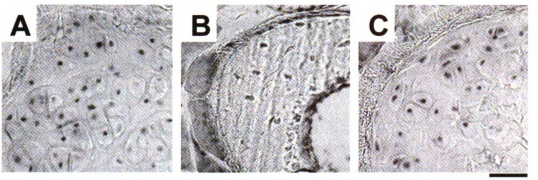

Figure 5. Photomicrographs of cross-sections through the three cartilages involved in dewlap extension: the second ceratobranchials (A), first ceratobranchials (B), and ceratohyals (C). Androgen receptor immunoreactivity is present and dark in many of the nuclei in (A) and (C) but is absent or light in the nuclei in (B). The ribbon-like appearance of the first ceratobranchials in (B) reflects the ossified nature of this component of the dewlap system; the second ceratobranchials (A) and ceratohyals (C) are hyaline cartilage (13, 14). All tissue is from a castrated male treated with testosterone and housed in breeding environmental conditions. Scale bar = 30μm.

Renal sex segment cell height was larger in BS than NBS males ($t = 14.26$; $p < 0.01$) suggesting that these males indeed had higher levels of circulating androgens (Figure 6).

Experiment 2: Testosterone manipulation

Testosterone treatment significantly increased the percent of AR+ RPM nuclei in DAPI-labeled tissue ($F = 9.99$; $p < 0.01$), although this measure did not differ across season ($F = 1.19$; $p = 0.29$), and these variables did not interact ($F = 0.23$; $p = 0.64$; Figure 2). Similarly, testosterone treatment significantly increased the percent of AR+ nuclei in hematoxylin-labeled tissue ($F = 14.58$; $p < 0.01$) while the effect of season ($F = 0.25$; $p = 0.63$) and the treatment x season interaction ($F = 3.24$; $p = 0.08$) failed to reach significance (Table 1). As in Experiment 1, the percent of AR+ nuclei was significantly different among nucleus types ($F = 11.20$; $p < 0.01$); centralized and edge nuclei had higher percentages of AR+ nuclei compared to those outside RPM fibers (both $p < 0.05$). Total number of RPM muscle fibers did not differ between groups (287-388 fibers on average; effect of season: $F = 3.79$; $p = 0.06$; effect of testosterone: $F = 0.55$; $p = 0.47$; interaction: $F = 0.0001$; $p = 0.99$). The percent of AR+ motoneurons did not differ across season ($F = 0.72$; $p = 0.41$) or with testosterone treatment ($F = 0.17$; $p = 0.69$), and these variables did not significantly interact (all means between 71.6 – 77.7%; $F = 1.55$; $p = 0.23$; Figure 3). As with Experiment 1, AR immunoreactivity was present in all components of the hemipenes (Figure 4) but did not obviously differ across groups.

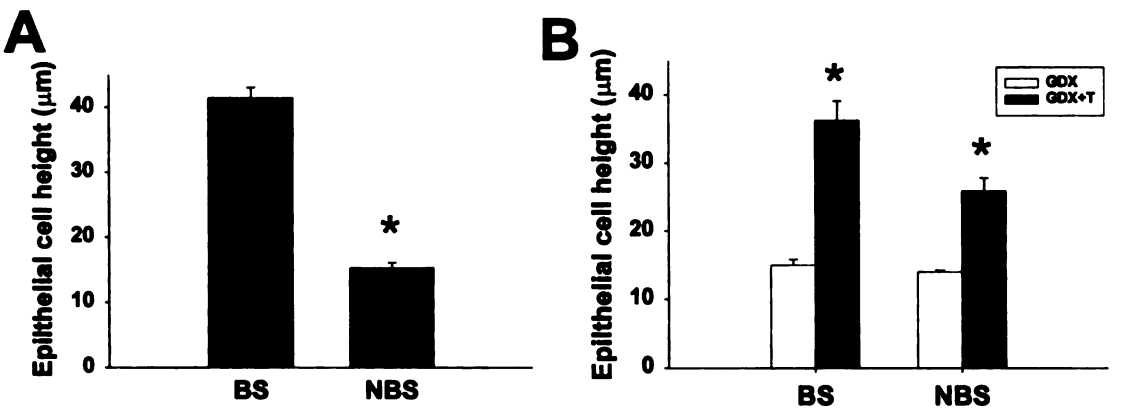

Figure 6. Mean (+/- SEM) cell height (µm) of epithelial cells in the renal sex segment in (A) gonadally intact males (grey bars) and (B) castrated males (GDX) treated with testosterone (T; black bars) or an empty capsule (white bars). Animals were housed either in breeding (BS) or non-breeding (NBS) environmental conditions. Asterisks indicate a statistically significant difference between BS and NBS males in (A) and a main effect of testosterone treatment in (B). A statistically significant main effect of season, as well as a significant season x testosterone interaction, is also present in (B).

In contrast to the RPM, testosterone treatment did not alter the percent of AR+ DAPI labeled nuclei in the CH muscle ($F = 0.17$; $p = 0.90$; Figure 2). Season also did not alter this measure ($F = 0.003$; $p = 0.96$), and the treatment x season interaction was not statistically significant ($F = 0.24$; $p = 0.63$). These results were replicated with hematoxylin and eosin stained tissue (Table 1), where neither testosterone treatment ($F = 1.71$; $p = 0.20$) nor season ($F = 0.51$; $p = 0.48$) altered AR immunoreactivity in the CH. The treatment x season interaction also failed to reach significance ($F = 0.24$; $p = 0.63$). While the percent AR+ nuclei did not differ among CH nucleus types in Experiment 1, the percent of AR+ nuclei was significantly different among nucleus types ($F = 4.30$; $p = 0.02$) in Experiment 2; the percent of AR+ centralized nuclei was higher than AR+ nuclei outside of CH muscle fibers ($p < 0.05$). Total number of CH muscle fibers did not differ between groups (300-318 fibers on average; effect of season: $F = 0.73$; $p = 0.40$; effect of testosterone: $F = 0.08$; $p = 0.78$; interaction: $F = 0.04$; $p = 0.85$). As in Experiment 1, AR immunoreactivity was present in both the first and second ceratobranchial cartilages, was very faint in the ceratohyals (Figure 5), and did not obviously differ between groups.

Renal sex segment cell height was significantly larger in breeding than non-breeding males ($F = 6.55$; $p = 0.02$) and in testosterone-treated males than castrated controls ($F = 54.89$; $p = 0.001$). Season and hormone treatment did significantly interact ($F = 4.38$; $p = 0.048$) whereby testosterone increased cell height more in breeding than non-breeding males (Figure 6).

DISCUSSION

Summary

The present results demonstrate that, in addition to the target tissues (dewlap cartilages and hemipenes), a large percentage of nuclei in the dewlap (CH) and copulatory (RPM) muscles express AR. Expression was consistent across season in both muscles in gonadally intact males (Experiment 1) as well as in castrated males treated with testosterone or vehicle (Experiment 2). Testosterone increased the percentage of AR+ nuclei in the RPM, but not the CH, regardless of season. In contrast to the dewlap motoneurons, which express relatively little AR protein or mRNA (approximately 44% of the cells in Amb X and none in Amb IX/VIImv; Rosen et al., 2002), the majority of T17-S1 motoneurons exhibit AR immunoreactivity. Yet, neither season nor testosterone manipulation altered the percentage of AR+ cells in this population.

Comparison to testosterone effects on morphology

While testosterone is the most potent hormone in the activation of both male courtship and copulatory behaviors in this species (Mason and Adkins, 1976; Crews et al., 1978; Adkins and Schlesinger, 1979; Winkler and Wade, 1998; Rosen and Wade, 2000), it has disparate effects on the underlying sexually dimorphic structures, the muscles and peripheral tissues in particular. We do not yet know what factors contribute to sex differences in the motoneurons in either system. However, testosterone increases RPM fiber and hemipenis size in the adult copulatory neuromuscular system (Holmes and

Wade, 2004b; Chapter 2), and androgens stimulate the survival of these structures before hatching (Holmes and Wade, in press; Chapter 5). In the dewlap system, testosterone manipulation does not appear to alter CH muscle fiber size in adult males (O'Bryant and Wade, 1999). However, androgens are involved in the masculinization of the CH muscles and second ceratobranchial cartilages in juveniles (Lovern et al., 2004b; Chapter 4). AR expression should be investigated during development, but the present results from adults are consistent with the idea that the morphological changes may involve a testosterone-induced increase in AR expression. Testosterone only increases RPM fiber size and hemipenis size when animals are housed in breeding environmental conditions (Holmes and Wade, 2004b; Chapter 2), although the hormone increases AR expression in both breeding and non-breeding animals, suggesting that other variables likely also contribute to the morphological effects (see below).

Taken together, these data suggest a variety of possible mechanisms that might mediate the effects of testosterone on the expression of courtship and copulatory behaviors in this species. In addition to the role the hormone plays in enhancing or maintaining structure of the peripheral copulatory system (likely via up-regulation of AR), it may also act on the receptors that are abundant in the limbic forebrain (Rosen et al., 2002) to facilitate motivation to display the behaviors (Morgentaler and Crews, 1978; Greenberg et al., 1984). Testosterone may also directly influence the execution of courtship and/or copulation by modulating the functions of the motoneurons in either system as well as the CH

muscle, in the absence of morphological changes in these particular structures. Electrophysiological and neurochemical studies would begin to address these issues. As the motoneurons, hemipenes and RPM all express AR, it will also be important to determine the site(s) of action for testosterone-induced changes in morphology of the copulatory muscles and target structures. While testosterone may act directly on RPM fibers to exert its trophic effects, other mechanisms may be involved. For example, this growth may instead be a response to testosterone directly increasing the size of the hemipenes, or it may be that the motoneurons somehow exert an anterograde effect on the muscles even though they do not show an increase in soma size themselves.

Comparison to other species: muscle

AR is commonly expressed in vertebrate muscle fibers, particularly in muscles that are sexually dimorphic (Monks et al., 2004; Dorlochter et al., 1996; Fischer et al., 1995; Veney and Wade, 2004). The present data demonstrate that testosterone treatment increases AR expression in lizard muscle fibers that exhibit morphological sensitivity to testosterone. While this testosterone-induced increase in the percent of AR+ nuclei in adulthood is consistent with previous reports in rats (Monks et al., 2004) and humans (Sinha-Hikim et al., 2004), a direct link between AR expression and morphological responsiveness is less clear across species. In male *Xenopus,* widespread AR immunoreactivity is detected in skeletal muscle fibers, including those that do not change in size in response to testosterone manipulation (Dorlochter et al., 1996). Furthermore, in

the sexually dimorphic and androgen sensitive larynx, three weeks of androgen treatment in adulthood causes a decrease in AR mRNA expression (Fischer et al., 1995), although expression of AR protein and mRNA are not always complementary (e.g., Rosen et al., 2002). Finally, while testosterone does increase AR immunoreactivity in rat muscles that exhibit morphological responsiveness to androgens, it also does so in those that do not (Monks et al., 2004). For both the levator ani (increases in size in response to testosterone) and extensor digitorum longus (does not change in size in response to testosterone), the percent of AR+ nuclei is increased by testosterone relative to castrated control males in both myonuclei and fibroblasts (Monks et al., 2004). However, as in rats, the effects of testosterone were consistent across different types of nuclei for both CH and RPM muscles in green anoles in the present experiment. AR expression did not change in any of the three classes of nuclei in the CH muscle but was increased by testosterone in all of them in the RPM muscle, suggesting that multiple cells types are responsive to androgens in this muscle and may contribute to the structural and/or functional changes induced by these steroids. Taken together, these data suggest that sensitivity of a given muscle to testosterone is not simply dictated by the distribution of AR. While functional AR are clearly required for androgen-induced effects (reviewed in Breedlove et al., 2002), presence of the AR protein does not necessarily confer morphological responsiveness to testosterone to a given cell.

Comparison to other species: central nervous system

AR is also commonly expressed in both the brain and spinal cord, and, as in muscle, androgens often increase AR expression in reproductive neural circuits. For example, testosterone increases AR expression in the medial preoptic nucleus, medial amygdala, lateral septum, and bed nucleus of the stria terminalis (posteromedial subdivision) in hamsters (Meek et al., 1997), in limbic system extracts (detected in Western blots) in mice (Lu et al., 1999), as well as in sexually dimorphic motoneurons in frogs (Perez and Kelley, 1996) and rats (Freeman et al., 1995). In the present report, a large portion (approximately 70-80%) of the T17-S1 motoneurons were AR+, although testosterone manipulation did not alter the percentage of these cells expressing the protein. As stated above, this lack of change in immunoreactivity parallels the fact that testosterone does not alter the soma size of these cells in this species. Either this population of motoneurons is truly unresponsive to testosterone, which seems unlikely, or the receptors are involved with functions in these cells other than changes in soma size. This dissociation between AR expression and morphological responsiveness to testosterone is similar to what occurs in a subset of the motoneurons in the spinal nucleus of the bulbocavernosus and dorsolateral nucleus of the rat copulatory neuromuscular system. Both nuclei are mixed populations in which a portion of cells exhibits testosterone-induced increases in soma size while the remaining cells do not (Collins et al., 1992). Motoneurons projecting to the bulbocavernosus, levator ani, and ischiocavernosus muscles increase in size in response to testosterone (Collins et al., 1992) and approximately 95% of these cells are AR+ (Jordan, 1997). However, the

remaining motoneurons project to the external anal sphincter and external urethral sphincter and these cells do not exhibit morphological responsiveness to testosterone, yet the majority of them also express AR (70% for the external anal sphincter and 90% for the external urethral sphincter; Jordan, 1997).

While we did not see any effect of season on AR in lizard copulatory motoneurons, some precedent exists for neural AR expression to fluctuate in concert with naturally occurring changes in testosterone levels. AR mRNA is distributed in several brain regions important for reproductive behaviors (including the medial preoptic area, external nucleus of the amygdala, and the ventromedial hypothalamus) in male and female leopard geckos (Rhen and Crews, 2001). Testosterone mediates both male and female reproductive behaviors in this species (Rhen et al., 1999), and AR mRNA expression in the female brain is increased during late vitellogenesis, when females are receptive and plasma testosterone levels are more than an order of magnitude greater than previtellogenesis (Rhen et al., 2000; Rhen et al., 2003). Similarly, increased androgen receptor expression is seen in the brains of goldfish (Pasmanik and Callard, 1988), Atlantic croaker (Sperry and Thomas, 1999), and Gambel's white-crowned sparrows (Soma et al., 1999), corresponding to time points with naturally occurring increased circulating testosterone.

Conclusions

AR immunoreactivity can, but does not necessarily, correspond to the effects of androgens on morphology within a given tissue. Testosterone up-

regulates AR expression in RPM muscle fibers, which increase in size in response to this steroid (Holmes and Wade, 2004b; Chapter 2). Yet, a high percentage of T17-S1 motoneurons (approximately 70-80%), as well as CH muscle fibers (approximately 60-70%), which do not appear to change in size in response to testosterone (O'Bryant and Wade, 1999), express AR. These structures may certainly be sensitive to testosterone in additional physiological and/or morphological capacities that have not yet been investigated, but it is clear that the expression of AR does not alone determine whether morphological change will occur in response to testosterone. Additional factors must contribute to the effects of androgens on these systems in adulthood. Taken together, not only are the morphological effects of androgens often complex both within and across species, but understanding the site of action of these hormones involves more than simply characterizing the distribution of AR. Ultimately, insight into the general principles of androgenic effects on the structure and function of sexually dimorphic circuits will come both from characterizing the distribution of AR in the tissues of interest and identifying how additional transcription factors, including steroid hormone receptor co-activators, are involved.

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

INTRODUCTION

Sexual dimorphism in behavioral function and associated morphological structure is a hallmark of reproduction in gonochoristic vertebrates. For example, in species such as plainfin midshipman fish (*Porichthys notatus*; Brantley et al., 1993a; Brantley and Bass, 1994; Knapp et al., 1999), African clawed frogs (*Xenopus laevis*; Sassoon and Kelley, 1986; Kelley and Dennison, 1990; Kelley and Tobias, 1999), and zebra finches (*Taeniopygea guttata*; Bottjer et al., 1985; Wade et al., 2002), adult males produce courtship vocalizations that are not produced by females, and the neuromuscular systems controlling them show male-biased dimorphisms that begin differentiating in development. Similarly, the neuromuscular system controlling penile function during copulation in rodents shows a number of male-biased dimorphisms that become apparent shortly after birth (Cihák et al., 1970; Breedlove and Arnold, 1980; Nordeen et al., 1985).

Often, gonadal steroids play a direct role in regulating the differentiation of sexually dimorphic neuromuscular systems as well as their peripheral, target tissues (e.g., reviewed in Cooke et al., 1998; Breedlove et al., 2002). Androgen production by juvenile male African clawed frogs increases after metamorphosis, which results in masculinization of components of the male song system including increased motoneuron and muscle fiber number (Kelley, 1986; Sassoon and Kelley, 1986; Kay et al., 1999). Similarly, just after birth, male rats produce androgens that masculinize the copulatory neuromuscular system by preventing cell death of the motoneurons and muscle fibers targeting the penis (Breedlove and Arnold, 1980, 1983a, b; Nordeen et al., 1985). However, early

exposure to testosterone (T) or its metabolites may not be necessary or sufficient for the sexual differentiation of some neuromuscular systems. The role of steroids in sexual differentiation of the zebra finch song system appears quite complicated (reviewed in Arnold, 1997; Wade, 2001). For example, during development androgens can masculinize motoneuron soma size and syrinx (the vocal organ) weight in females, and estrogens can feminize the syrinx in males (Wade et al., 2002). However, the antiandrogen flutamide does not prevent masculinization, and the aromatase inhibitor fadrozole does not prevent feminization (Wade et al., 2002). Thus, the neuromuscular systems involved in courtship and copulation are frequently sexually differentiated in development, but comparative study suggests that the underlying mechanisms that regulate this differentiation may be diverse.

In the present study, we examine the development of neuromuscular systems and target tissues involved in courtship and copulation in the green anole, *Anolis carolinensis*. This lizard has a polygynous social organization, breeding seasonally from approximately April through July, with females laying single-egg clutches over the entire four month breeding season (see Lovern et al., 2004a for natural history overview). Hatchlings emerge during the summer and early fall months and reach sexual maturity in the following breeding season, approximately 6-9 months later. Both adult males and females communicate via stereotyped head bobbing displays that may be given with or without the extension of a red throat fan called a dewlap (DeCourcy and Jenssen, 1994; Jenssen et al., 2000). Males use these head bobbing displays with dewlap

extension in territorial advertisement and in aggressive and sexual interactions, whereas females use them in aggressive (with dewlap extension) and sexual (without dewlap extension) interactions and overall at much lower rates (Greenberg and Noble, 1944; Nunez et al., 1997; Jenssen and Nunez, 1998;). During courtship, the male approaches the female while head bobbing and prominently displaying his extended dewlap. If the female is receptive, she remains stationary and gives head bob displays in return – but without dewlap extension – often adopting a characteristic neck-bend posture (e.g., Crews, 1980). The male then bites the female on the back of the neck as he mounts and intromits one of two bilateral, independently controlled penises (called hemipenes), characteristic of lizards and snakes (Pough et al., 2001).

The anatomy involved in dewlap extension, like the courtship behavior described above, is sexually dimorphic. Two pools of motoneurons in the caudal brainstem regulate dewlap extension. One is located in the vagal portion of nucleus ambiguus (AmbX), and the other in the glossopharyngeal region of nucleus ambiguus and the ventral motor nucleus of the facial nerve (AmbIX/VIImv) (Font, 1991; Wade, 1998). These motoneurons innervate the bilaterally symmetrical ceratohyoid muscles, which upon contraction cause the 2nd ceratobranchial cartilage to bow out, extending the dewlap (Bels, 1990; Font and Rome, 1990). Extended dewlap area is approximately 7-fold greater in adult males than in adult females (Jenssen et al., 2000). Underlying this dramatic sex difference are male-biased dimorphisms in 2nd ceratobranchial cartilage length, ceratohyoid muscle weight and fiber cross-sectional area and number,

neuromuscular junction size, motoneuron soma size, and nerve cross-sectional area (Wade, 1998; O'Bryant and Wade, 1999, 2002b).

Similar to the anatomy involved in dewlap extension, the hemipenis neuromuscular system is highly sexually dimorphic. Each hemipenis is controlled by motoneurons in the ipsilateral trunk segment 17 and sacral segment 1 of the caudal spinal cord (T17-S1) (Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1). These motoneurons innervate the paired transversus penis (TPN) muscles, which evert the hemipenes, and the retractor penis magnus (RPM) muscles, which retract them (Arnold, 1984; Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1). In adults, the hemipenes, TPN, and RPM are present only in males (Ruiz and Wade, 2002). The T17-S1 motoneurons are present in both males and females, due to the fact that a subset of the cells in this nucleus innervates targets possessed by both sexes (a leg muscle and the cloacal sphincter; Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1). Nevertheless, overall the motoneurons are larger and more numerous in males (Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1).

The dewlap system (i.e., the 2nd ceratobranchial cartilage, the ceratohyoid muscles, and AmbX and AmbIX/VIImv motoneurons) is sexually monomorphic at hatching. However, between 60 and 90 days post-hatching, both cartilage length and muscle fiber cross-sectional area have become larger in males, although motoneuron soma size has not (O'Bryant and Wade, 2001). This period of differentiation occurs when plasma T levels in juvenile males are elevated compared to females (Lovern et al., 2001), suggesting a potential causal

relationship. Less is known about sexual differentiation of the hemipenis system (i.e., the hemipenes, TPN and RPM muscles, and T17-S1 motoneurons). In lizards, both males and females develop hemipenes as embryos, but they regress in females prior to hatching (e.g., Dufaure and Hubert, 1961). The muscles and motoneurons have not been examined during development.

The objective of the present study was to determine the effect of juvenile T manipulation on the dewlap and hemipenis systems in green anoles. Post-hatching males first have plasma T levels that are significantly higher than those of females at approximately 30 days of age (Lovern et al., 2001). Thus, we manipulated juvenile T exposure at this time by giving gonadally intact females either blank or T implants, and by castrating or sham-castrating juvenile males. Then, at 90 days post-hatching, we euthanized all juveniles and assessed treatment effects on both the dewlap and hemipenis systems. We predicted that females given T implants would develop larger, more masculine, dewlap systems compared to females given blank implants, and that castrated males would develop smaller, more feminine, dewlap systems compared to sham-castrated males. As described above, the hemipenes are sexually differentiated by hatching, much earlier than the dewlap system. However, whether this differentiation is reversible was unknown, and the associated muscles and motoneurons had not been examined during development. Therefore, in addition to investigating the effects of T on the sexual differentiation of the dewlap neuromusculature, we addressed the degree to which steroid-mediated plasticity may exist in the hemipenis system.

MATERIALS AND METHODS

Animals and housing

Our established laboratory breeding colony provided us with juveniles for this study. Briefly, adult (≥ 50 mm snout-vent length, SVL) *A. carolinensis* were purchased in the breeding season (Charles Sullivan Co., Nashville, TN) and kept in the laboratory in cages consisting of one male and 3-7 females under conditions conducive to breeding (e.g., Lovern et al., 2004a). Breeders were kept on a 14:10 h light:dark cycle using a combination of fluorescent, ultraviolet, and incandescent lights. Ambient temperature ranged from 18°C at night to 28-38°C during the day depending on location in each cage relative to a basking light. Relative humidity was set at 70%. Nest boxes were placed into each cage and checked daily for eggs, which were incubated individually in sealed plastic cups containing a 1:1 vermiculite:dH_2O mixture. Incubation temperature averaged 28°C (range 27-29°C); hatching occurred at a mean of 34.1 days (SE=0.6).

Hatchlings were sexed by post-anal scale size (males have two enlarged post-anal scales, females do not), their SVL and mass were recorded, and they were toe-clipped for permanent individual identification. Groups of up to 20 juveniles were housed in 110 L aquaria furnished with a peat moss substrate and multiple dowels, rocks, and artificial vegetation for perching, basking, and hiding. The environmental conditions were the same as above and approximate those found in the field during the summer. Juveniles had access to water in shallow dishes *ad libitum*, their cages were misted daily, and they were fed daily a diet of vitamin-dusted crickets and vestigial-winged fruit flies.

Experimental design

Manipulations were conducted according to NIH guidelines and with approval of the Michigan State University All-University Committee on Animal Use and Care. At 30 days post-hatching, juveniles were again measured and weighed, after which they were treated in one of four ways. Females were given either blank or T implants and males were either castrated or sham-castrated. Implants consisted of T (testosterone propionate; Steraloids, Inc.) uniformly mixed into silicone sealant (Type A, Dow Corning). This mixture was expelled through a 1-cc syringe in a straight line onto wax paper and allowed to dry over night, after which it was cut into 3 mm lengths. Each implant contained approximately 1 mg of T. Blank implants were made in the same fashion, minus the addition of T.

Surgeries were performed following isoflurane anesthesia. In females, a small (~ 3 mm) dorsolateral incision was made in the skin, just rostral to the hind legs. Either a T or a blank implant was then inserted subcutaneously, and the incision was closed with Vetbond tissue adhesive (3M). In males, a dorsolateral incision (~ 3 mm) was made through the skin and body wall adjacent to where the testes were located. In castrates, the testes were removed; in sham-castrates, the testes were probed and left intact. These incisions were closed with silk sutures.

Following surgeries, the lizards were placed into cages identical to those in which they were housed for the first 30 days. They were kept in groups of up to 16 individuals, fed and watered as described above, and were mixed in the

enclosures across sex and treatment. Lizards were housed in this fashion until they reached 90 days post-hatching (see below). Every 14 days, we checked whether each female still had her implant, and replaced it when necessary (9 of 19 females required re-implantation over the 60 days of treatment). Survival to day 90 in females was 87% (11 of 13 T-implanted and 9 of 10 blank-implanted survived), and in males was 77% (10 of 15 castrated and 10 of 11 sham-castrated survived). One female with a T implant was not included in the study; her mass at 90 days was > 4 SEs below the female mean and her overall physical appearance clearly suggested that she was unhealthy.

Tissue collection and analysis

At 90 days post-hatching, SVL and mass were recorded for the third time for each juvenile, after which they were decapitated. Blood samples were collected from the trunk, and the plasma fractions obtained following centrifugation were measured to the nearest µL and individually stored at –80°C until analysis for T content (see below). The kidneys were removed and placed in Bouin's fixative. The epithelial cells of the renal "sex segments", analogous to the mammalian prostate, increase in height in the presence of androgens, and thus serve as a T bioassay (Cuellar et al., 1972). In males, we used a dissecting microscope to confirm that castrates had no testicular tissue, and that sham-castrates still had their testes. In all lizards, the 2nd ceratobranchial cartilage was exposed by removing the dewlap skin and measured with calipers to the nearest 0.1 mm. The lower jaw, containing the ceratohyoid muscles, was placed in

Bouin's fixative. The cranium, containing the brain tissue including the AmbX and AmbIX/VIImv motoneurons, was placed in 10% phosphate-buffered formalin. To assess potential effects in the hemipenis system, the ventral half of the rostral third of the tail (the region where the hemipenes and RPM muscles are located) and the trunk-sacral region of the spinal cord containing the T17-S1 motoneurons were placed in Bouin's fixative. The tail tissue also contained the caudifemoralis (CF), a leg muscle not directly involved in copulation, which was measured to assess the specificity of T action.

After fixing for five days, the brain was removed from the skull and, along with the other tissues, was soaked in 70% ethanol overnight, dehydrated, and cleared in xylene. Tissues were then individually embedded in paraffin and sectioned at 10 μm. Brains and spinal cords were stained with thionin, throats and tails were stained with trichrome, and kidneys were stained with hematoxylin and eosin.

Measurements were taken, blind to treatment, using NIH Image software on a PC and an Olympus BX-60 light microscope. In the dewlap system, cross-sectional area was assessed in 15 ceratohyoid muscle fibers for each animal, randomly selected across the left and right muscles. Soma size was measured in 10 AmbX and 10 AmbIX/VIImv motoneurons from each side (20 measurements per animal) when possible, although in a few individuals fewer or no measurements were obtained from one side. These cells are easily distinguished based on not only their location, but also on the size relative to neighboring neurons and specific morphological characteristics (Wade, 1998). Hemipenis

cross-sectional area was assessed in 10 sections per animal, spaced approximately 50μm apart, for each the left and right hemipenes (20 measurements total). RPM muscle fiber cross-sectional area was measured in 25 fibers in each the left and right muscle for every animal. The same procedure was followed for the CF muscle. Cross-sectional areas of TPN muscle fibers could not be analyzed because those fibers run parallel to plane of sectioning (i.e., perpendicular to those of the RPM and CF; Ruiz and Wade, 2002); however their presence/absence was noted. Twenty measures of cross-sectional area, from each the left and right pools of T17-S1 motoneurons, were collected for each animal. As for the dewlap motoneurons, the anatomical location and particular characteristics of size, shape and orientation make these neurons easy to identify (Ruiz and Wade, 2002). T17-S1 motoneuron number was also estimated because it is increased in adult males compared to females (dewlap motoneuron number is sexually monomorphic). Motoneurons with an in-focus nucleolus were counted in every fifth section throughout the rostro-caudal extent of the T17-S1 nucleus and multiplied by the sampling ratio. Finally, epithelial cell height was measured in four cells in each of four tubules in the renal sex segment, for a total of 16 measurements per animal. A mean was computed for each size measure in each animal for statistical analysis (see below). The estimated total motoneuron number was analyzed after computing a mean for the left and right sides. Sample sizes vary from 7 to 10 across tissue type (see table and figures) due to histological artifact.

Radioimmunoassay

Plasma levels of androgen were measured by ether extraction and subsequent radioimmunoassay (RIA). Samples were thawed and mixed with 0.5 mL dH_2O to provide sufficient volume for extraction, and were equilibrated overnight at 4°C with 1000 cpm of ^{3}H-T (NET-370, 95 Ci/mmol) from Perkin Elmer for individual recovery determinations. The following day, samples were extracted twice with 3 mL diethyl ether, dried under nitrogen gas, and reconstituted in 0.1 M phosphate buffered saline. They were then stored overnight at 4°C. A competitive binding RIA was performed using ^{3}H-T and antibody from Wien Laboratories (T-3003). Because this antibody reacts approximately equally with T and dihydrotestosterone, our measure is an assessment of circulating androgen rather than strictly T. The standard curve ranged from 1 to 250 pg and was run in triplicate. Samples were run in duplicate, averaged, and adjusted for individual recovery and initial sample volume. The intra-assay coefficient of variation, based on four aliquots from a standard pool included in the assay, was 4.2%.

Data analysis

Data were analyzed separately for males and females because the treatments were not parallel across the sexes. Nonparametric statistics were used to analyze total androgen (Wilcoxon rank sum and Spearman rank correlation tests) because these data were not normally distributed. Most of the measures of neuromuscular structures and their and peripheral targets showed

significant positive relationships with SVL (Pearson correlations, $P<0.05$). Therefore, prior to further statistical analysis we divided each of these data points by the SVL of the animal from which it was collected (motoneuron number data were not corrected). The data followed a normal distribution and were analyzed using ANOVAs or two-sample t-tests. The residuals from regression plots of SVL vs. motoneuron and muscle fiber area, cartilage length and hemipenis area were also analyzed with t-tests. The statistical significance of the results differed in only one case. It is indicated below; otherwise, for simplicity, only the analyses of the ratios are reported. Hypothesis tests were two-tailed with an overall $\alpha = 0.05$; data are presented as means $\pm$ SE.

RESULTS

Body length and mass

Body length and mass increased steadily and significantly for both females and males. On average, females ranged from 23mm and 273mg on the day of hatching to 35mm and 947mg at day 90 (SVL: $F_{2,56}=467.7$, $P < 0.0001$; mass: $F_{2,56}=187.6$, $P < 0.0001$). Similarly, males grew from 23mm and 284mg to 35mm and 986mg (SVL: $F_{2,59}=238.1$, $P < 0.0001$; mass: $F_{2,59}=142.9$, $P < 0.0001$). However, there was no effect of treatment on either SVL or mass, nor were there treatment x SVL or treatment x mass interactions, in either females or males.

Treatment effects on T

Total androgen levels were significantly higher, and epithelial cells in the renal sex segments were significantly larger, in juvenile females given T implants than in those given blank implants (androgen level: W=61, P=0.02; epithelial cell height: t=5.39, P<0.001; Table 1). Furthermore, a significant positive relationship between androgen level and epithelial cell height was detected in females (Spearman's r=2.48, P=0.01). Similarly, mean androgen level was higher and mean epithelial cell height was greater in sham-castrated than in castrated juvenile males (Table 1). Although these mean differences were in the expected direction, they were not statistically different (androgen level: W=125, P=0.14; epithelial cell height: t=1.60, P=0.13), and the positive relationship between androgen level and epithelial cell height only approached statistical significance (Spearman's r=1.66, P=0.09). Nevertheless, it is clear that androgen differences in males were sufficient to produce treatment effects (see below), even if group means for measures of T exposure were too close to be statistically distinguished.

Sex	Androgen (ng/ml)	Epithelial cell height (μm)
Treatment (N)		
Female		
Blank (9)	0.77 (0.23)	14.1 (0.54)
T (10)	18.34 (5.5)*	28.1 (2.6)**
Male		
Sham (10)	1.87 (0.47)	16.0 (0.65)
Castrated (10)	1.02 (0.21)	14.3 (0.88)

Table 1. Mean (SEM) plasma androgen levels and epithelial cell heights of the renal sex segments of juvenile male and female green anoles. * $p < 0.05$. ** $p < 0.001$.

Dewlap system

Females with T implants had longer 2nd ceratobranchial cartilages (t=5.47, P<0.001; Figure 1) and larger ceratohyoid muscle fibers (divided by SVL: t=2.62, P=0.02; residuals: t=1.90, P=0.08; Figures 1 and 2) than did females with blank implants. Sham-castrated males had longer 2nd ceratobranchial cartilages (t=2.33, P=0.03) than did castrated males (Figure 1), but an effect of treatment on ceratohyoid muscle fibers was not detected (Figures 1 and 2). Soma sizes of AmbX and AmbIX/VIImv motoneurons were not affected by treatment in either females or males (Figure 1).

Hemipenis system

Females did not develop hemipenes in response to T exposure, nor did they develop RPM or TPN muscles (Figure 3). In males, castration resulted in significantly smaller hemipenes (t=3.08, P=0.007; Fig. 3 and 4), but had no effect on RPM muscle fiber size (Figure 4). The CF muscle, measured as a control, contained significantly *smaller* fibers in T-implanted than in blank-implanted females (t=3.18, P=0.006), but showed no size differences across male treatment groups (data not shown). A decrease in CF muscle fiber size also occurs in response to T treatment in adult males (Holmes and Wade, 2004), but the mechanisms are unknown. As in the dewlap system, treatment did not affect soma size for T17-S1 motoneurons in females or males (Figure 4). It also did not alter motoneuron number in females (blank [n=9]: 232.5±8.0; T[10]: 247.3±12.4) or males (castrated [10]: 236.8±13.5; sham [10]: 245.5±10.4).

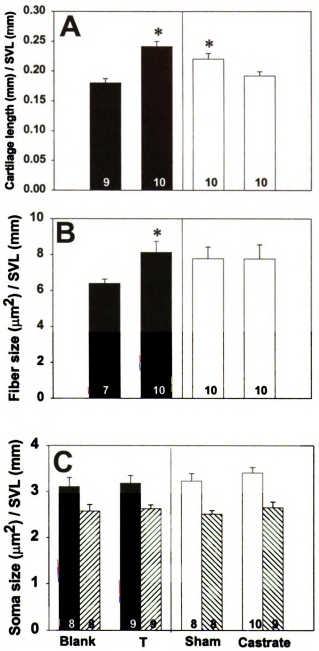

Figure 1. Treatment effects in juvenile female and male green anoles on morphological components responsible for dewlap extension. (A) Length of the 2nd ceratobranchial cartilage; (B) cross-sectional area of the ceratohyoid muscle fibers; (C) cross-sectional areas of AmbIX/VIImv (solid bars) and AmbX (hatched bars) somata. Data were sampled 90 days after hatching, following 60 days of treatment, and are presented as mean + SE (corrected for snout-vent length, SVL). Sample sizes are given at the bottom of the bars and are not identical for each tissue type due to histological artifact. Male and female data sets were analyzed separately by two-sample t-tests. *$P < 0.05$.

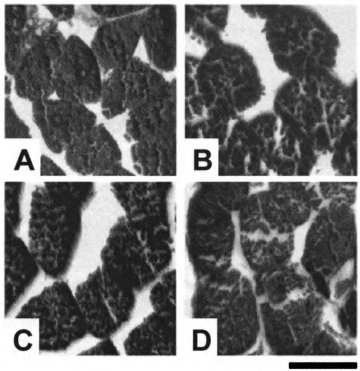

Figure 2. Photomicrographs of ceratohyoid muscle fibers collected from juvenile green anoles 90 days after hatching, following 60 days of treatment. (A) Blank-implanted females had significantly smaller fibers than (B) testosterone-implanted females; (C) sham-castrated and (D) castrated males did not differ significantly. Scale bar = 20 μm.

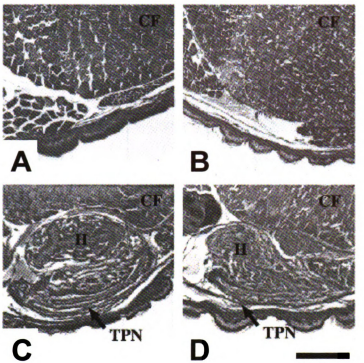

Figure 3. Photomicrographs of the tail region in juvenile green anoles where hemipenes are located, collected 90 days after hatching, following 60 days of treatment. (A) Blank-implanted and (B) testosterone-implanted females did not develop hemipenes; (C) sham-castrated males had significantly larger hemipene cross-sectional areas than (D) castrated males. Scale bar = 20 μm. CF = caudifemoralis; HP = hemipene; TPN = transversus penis.

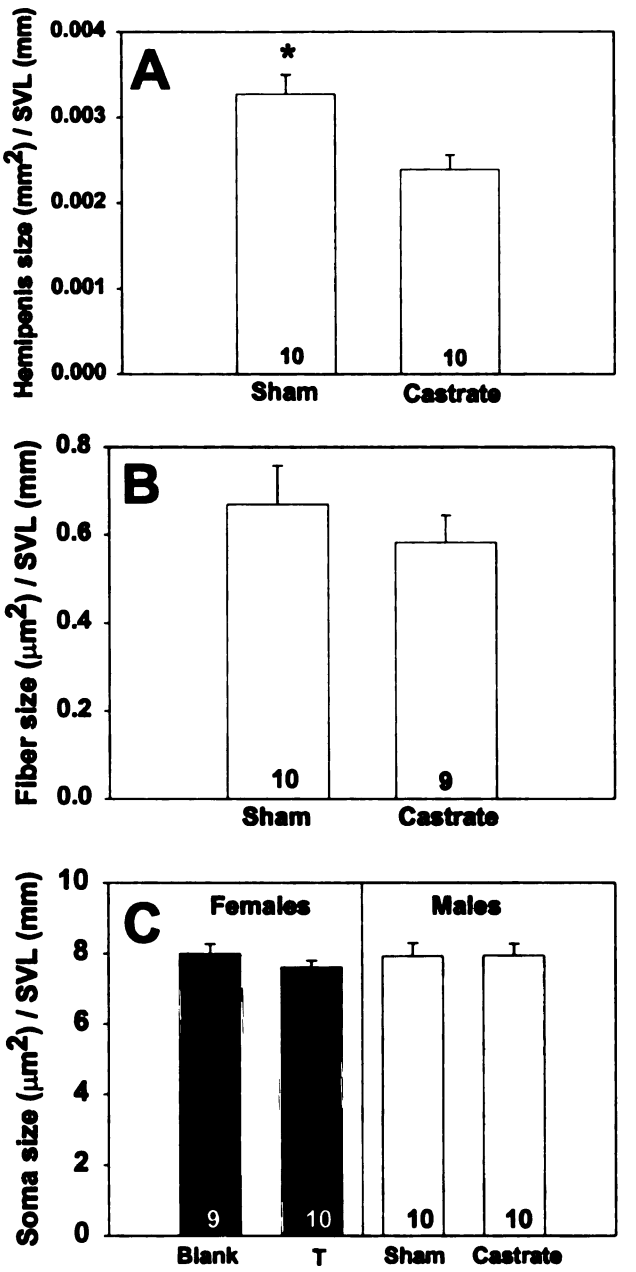

Figure 4. Treatment effects in juvenile female and male green anoles on hemipenes and associated neuromusculature sampled 90 days after hatching, following 60 days of treatment. (A) Hemipene and (B) retractor penis magnus (RPM) fiber cross-sectional areas in males; females did not develop hemipenes or RPMs. (C) Cross-sectional area of T17-S1 motoneuron somata in females and males. Data are presented as mean + SE, corrected for snout-vent length, SVL. Sample sizes are given at the bottom of the bars and are not identical for each tissue type due to histological artifact. Male and female data sets were analyzed separately by two-sample t-tests. *$P < 0.05$.

DISCUSSION

Summary

Our data suggest that steroid-mediated plasticity is present during juvenile development in neuromuscular systems and their target tissues (cartilage and hemipenes) involved in courtship and copulation in green anoles. As predicted, juvenile females given T implants developed longer dewlap cartilages and larger dewlap muscle fibers than did those given blank implants. Similarly, castrated juvenile males developed shorter dewlap cartilages than intact males, although dewlap muscle fiber size was not affected by treatment. The effect of T manipulation did not extend to dewlap motoneuron soma size in either males or females. Collectively, these results suggest that the peripheral tissues, but not the motoneurons in the dewlap system, differentiate during juvenile development and that T likely mediates this process. With regard to the hemipenis system, none of the juvenile females in either the T or control group possessed hemipenes or the underlying muscles controlling them, nor were there any effects on motoneuron soma size or number. These results suggest that sexual differentiation of the hemipenis system is largely complete by post-hatching day 30, when our treatments began. Consistent with that idea, castration of males did not affect muscle fiber size or motoneuron soma size or number, but it caused a reduction in hemipenis size. Thus, from post-hatching days 30 to 90, beyond the period of sexual differentiation, testis-mediated plasticity exists in the copulatory peripheral tissue, but its neuromuscular effectors are less or not responsive during this period.

Questions raised by the present data

Two general classes of questions are suggested by the present data. The first set involves mechanisms of action for sexual differentiation; the second concerns the timing of responsiveness to testosterone and how the morphological changes in the dewlap and hemipenis systems relate to organizational vs. activational theories of steroid hormone action.

Mechanisms of sexual differentiation. Juvenile T clearly masculinizes the dewlap system cartilage. Administering the hormone to females enlarged the structure, and castrating males reduced it. However, the muscle fibers were affected more modestly and only in females. Why? The diminished response might be due to a difference in any number of factors influencing androgen action (e.g., steroid metabolism, receptor distribution). Likely, however, the differential effect between the sexes is due to the manner in which T was manipulated in males and females (castration vs. implants). Plasma T was approximately 20 times higher in treated compared to control females (and similar to that of breeding adult males; Jenssen et al., 2001), whereas castrated males had approximately half the plasma T of sham-castrates (Table 1). The implants may have provided sufficient T exposure to induce dewlap muscle growth in females, while castrations did not sufficiently reduce T to prevent muscle growth in males. The relative contributions of the testes and other organs to circulating androgen levels are unknown for developing anoles; substantial adrenal output is a reasonable possibility. Future work could circumvent this issue by treating

juvenile males with anti-androgens, which should inhibit muscle growth if T is responsible.

The hemipenis system is more completely differentiated than the dewlap system. Adult males have hemipenes and associated musculature; females do not. The sex difference in hemipenes at least is obvious by the day of hatching (Holmes and Wade, unpublished), and T treatment from post-hatching days 30-90 does not reverse this effect. When in embryonic development does differentiation occur and is it mediated by T? Little work in this area has been done in any lizard species. In *Lacertids*, estradiol feminizes development of genital structures, but opposite to anoles, these animals exhibit female heterogamity (reviewed in Adkins-Regan, 1981; Raynaud and Pieau, 1985). Treatment of "post-hatching" (timing undefined) or adult male anoles with estradiol causes atrophy of the hemipenes (Forbes, 1964), but that could be due to decreased testicular function caused by negative feedback. The developmental time course needs to be worked out and the steroid environment of embryonic anoles needs to be manipulated in future experiments.

Does T influence sexual differentiation of either the copulatory or dewlap motoneurons? Although dewlap motoneurons are larger in adult males than females (Wade, 1998; O'Bryant and Wade, 1999), their size has not yet differentiated by post-hatching day 90 (O'Bryant and Wade, 2001). It is possible that T influences this feature later in development, perhaps as the animals enter their first breeding season the following spring and males initially experience adult levels of T. Alternatively, T may not directly influence development of

dewlap motoneuron soma size; these cells may become dimorphic only after the dewlap has been used more by males (unlike adults, juvenile males and female extend their dewlaps equivalently; Lovern and Jenssen, 2001). Both T and T-mediated behavior affect motoneuron soma size in the copulatory system of rats, although increased use appears to decrease this measure (Breedlove, 1997a; but see Raouf et al., 2000). It is presently unknown when the copulatory motoneurons differentiate in unmanipulated green anoles, but it is likely after post-hatching day 90. While not completely appropriate to compare between these groups due to different types of surgical manipulations, soma size and cell number were equivalent in sham-castrated males and blank-implanted females in the present study. Thus, either scenario could apply to them as well. That is, the soma size and/or number of copulatory motoneurons may differentiate just prior to or during their first breeding season. All of the previous experiments on morphology of the dewlap and copulatory systems have been conducted in adults of unknown ages, collected from the field sometime after they have entered their first breeding season, so we have no way at present of addressing this issue.

Given that juvenile T has at least some masculinizing effects, where specifically does it act? Dewlap motoneurons (AmbX) contain androgen receptor (AR) in adulthood (Rosen et al., 2002), but it is unknown whether any of the other dewlap or copulatory tissues assessed in this study do, and AR distribution has not yet been investigated in development. Adult leopard geckos express AR in hemipenes (Rhen and Crews, 2001), and given the robust response of

hemipenis morphology to castration in the present experiment (even stronger than our standard renal sex segment bioassay), it is likely that juvenile anoles do as well. In theory, T could act on any of the structures to induce morphological change. Although there is some variability due to developmental stage, AR are expressed in both muscles and motoneurons in other sexually dimorphic courtship and copulatory systems (Arnold, 1980; Fishman et al., 1990; Jordan et al., 1991, 1997; Godsave et al., 2002; Breedlove et al., 2002; Veney and Wade, 2004). It is also possible that the androgen acts on the more peripheral structures only (dewlap cartilage and hemipenes) and the muscles and motoneurons are subsequently masculinized due to enhanced mechanical stimulation and/or retrograde chemical signals. Future work on the distribution of AR will be critical to addressing this question of where androgen acts. Additionally, that work may provide insight as to how the dewlap and copulatory systems differentially respond to T at several life stages.

Timing of T-responsiveness: Implications for the classic theory of organizational effects of hormones.

Steroid effects on early sexual differentiation and subsequent behavioral expression have classically been conceptualized by theories of 'organization' and 'activation' (Phoenix et al., 1959). However, it is increasingly clear that they represent extremes on a continuum of effects on nervous system and behavior (e.g., Arnold and Breedlove, 1985; Breedlove et al., 1999). Male-biased sex differences in courtship and copulatory behavior in green anoles are clearly

activated by T (e.g., Winkler and Wade, 1998; Rosen and Wade, 2000). But what do our data suggest about the role of T in the organization of morphology?

Some aspects of the dewlap system seem to fit a traditional organizational action of T. In particular, the cartilage and muscle at least appear to be permanently organized during development. Male-biased dimorphisms in neuromuscular structures of the dewlap exist at many levels (see Introduction), and these sex differences are quite stable; neuromuscular structure varies little between breeding and non-breeding seasons, and androgen manipulations have no effect on the sexual dimorphisms in this system in adults (O'Bryant and Wade, 1999, 2002b). Similarly, dewlap coloration is sexually dimorphic and fixed in adult tree lizards (*Urosaurus ornatus*), but like the anole dewlap structures, it is sensitive to androgen exposure in both juvenile males and females until around 60 days post-hatching (Hews and Moore, 1995, 1996; Hews et al., 1994). However, these effects on dewlaps of both species are quite late, especially considering that lizards hatch in very precocial states. They may be similar to what occurs during puberty in mammals (e.g., Romeo et al., 2002), when dramatic sex differences appear in a second, key organizational window during development, well beyond the perinatal period. A similar idea is suggested for the copulatory motoneurons of Mongolian gerbils (*Meriones unguiculatus*), which appear to develop later than those of other rodents (Fraley and Ulibarri, 2001). However, the late differentiation of the dewlap system may not be entirely like "classic" mammalian puberty, in which reproductive behavior and fertility immediately follow pubertal maturation, given appropriate environmental

conditions. In anoles, the increase in T to levels typical of breeding males, and consequent reproduction, does not occur until the spring following the summer of hatching, 6-9 months later. Perhaps it is therefore more appropriate to think of the dewlap and copulatory systems in anoles as having two distinct organizational periods prior to reproductive maturation.

The fact that juvenile T manipulations do not induce masculinization of the hemipenis system in females is consistent with the idea that it is also permanently organized, albeit much earlier in development. It is, of course, possible that factors other than testosterone could induce growth (or prevent regression) of this system in juvenile females. However, data from at least one other lizard species suggest that T is important in some of these structures. Adult female leopard geckos (*Eublepharis macularius*) retain the capacity to develop hemipenes in response to as little as two weeks of androgen treatment (Rhen et al., 1999). This result suggests that, in contrast to green anoles, the hemipenis system in leopard geckos may not be permanently organized, which may be fundamentally related to their mode of sex determination (leopard geckos have temperature-dependent sex determination, in contrast to anoles which have genotypic sex determination; Viets et al., 1994).

While juvenile female anoles apparently do not grow hemipenes, this system shows some plasticity in adulthood in response to T, similar to the copulatory system of rats in which both muscles fibers and motoneurons enlarge in response to adult T (reviewed in Breedlove et al., 2002). However, the sensitivity to androgen in adult anoles depends on the environmental conditions

(Holmes and Wade, 2004b; Chapter 2). That is, hemipenis and RPM fiber size are not different in intact males in the breeding and non-breeding seasons. But, castrated males housed in breeding conditions (long days and warm temperatures) respond to T treatment with dramatic increases in hemipenis and RPM fiber sizes, whereas males housed in non-breeding environmental conditions do not, indicating that sensitivity to T is greater in the breeding season.

This differential adult plasticity between the dewlap and copulatory systems parallels behavior to some degree. Like dewlap morphology, the frequency of its extension shows strong male-biased sex differences (e.g., Jenssen et al., 2000; Nunez et al., 1997). But, unlike the underlying structure, the behavior shows dramatic seasonal differences in *A. carolinensis* (Jenssen et al., 1995, 1996) and the congeneric *A. sagrei* (Tokarz et al., 2002), as well as effects due to androgen manipulation (Adkins and Schlesinger, 1979; Winkler and Wade, 1998; O'Bryant and Wade, 2002a; Tokarz et al., 2002) that are consistent with endogenous T profiles (Lovern and Wade, 2001; Lovern et al., 2001). Both males and females use their dewlaps more during the breeding season, when androgen levels are higher (approximately 20 and 0.25 ng/ml for males and females respectively), than during the non-breeding season, when androgen levels are lower (10 and 0.1 ng/ml, respectively). Importantly, however, while the behavior is displayed far less frequently, the dewlap *is* extended by adults of both sexes in the non-breeding season (Jenssen et al., 1996). That maintenance of behavior may require (or facilitate) stability of the underlying morphology. In

parallel, T-induced plasticity in the RPM and hemipenes only under breeding environmental conditions mirrors the fact that, unlike dewlap use, T can facilitate copulation during the breeding, but does not appear to in the non-breeding, season (O'Bryant and Wade, 2002a). As all juveniles in the present study were reared under summer environmental conditions (those that are conducive to breeding in adults), it is unclear whether temperature and/or photoperiod might affect responsiveness to T at this age.

Relationships between structure and function and between the role of T in behavioral facilitation and masculinization of morphology (in terms of both sexual differentiation and adult plasticity) are common (see Cooke et al., 1998; Breedlove et al., 2002). However, the *selective* plasticity across the two neuromuscular systems in green anoles indicates perhaps even more broad definitions of organizational and maybe activational effects of steroid hormones than previously considered. That is: (1) while sexual differentiation of both the dewlap and copulatory systems appears permanent, it is far more extreme in one than the other (females lack the hemipenis system, but have a reduced dewlap system); (2) sexual differentiation of the two systems occurs at quite different life stages (T in juvenile females masculinizes dewlap, but not the hemipenis, structures); and (3) the hemipenis, but not the dewlap, system shows plasticity in adulthood, but it is tempered by environmental conditions. The opportunity to simultaneously examine the effects of identical treatments on two neuromuscular systems required for the full suite of masculine reproductive behaviors in anoles will allow us to further characterize the possibilities for modulating structures and

their functions, and importantly to uncover the mechanisms involved in mediating specific aspects of the changes.

CHAPTER FIVE

Holmes MM, Wade J (2005). Sexual differentiation of the copulatory neuromuscular system in green anoles (*Anolis carolinensis*): normal ontogeny and manipulation of steroid hormones. J Comp Neurol: in press.

INTRODUCTION

Gonadal steroid hormones commonly control differentiation of sexually dimorphic neuromuscular systems. Androgens masculinize both the muscles and motoneurons in the rodent copulatory neuromuscular system during perinatal development (e.g., Breedlove et al., 1982; Breedlove and Arnold, 1983a, 1983b; Nordeen et al., 1985), while estradiol (E) increases both the soma size (Breedlove, 1997b) and dendritic outgrowth (Goldstein and Sengelaub, 1994) of motoneurons in this system. Similarly, androgens masculinize (increase) neuron, axon, and muscle fiber number in the laryngeal system of juvenile African clawed frogs (Sassoon and Kelley, 1986; Robertson et al., 1994; Kay et al., 1999), muscle fiber number in the sonic (vocal) muscle of juvenile plainfin midshipman fish (Brantley et al., 1993b), and they can affect syrinx weight and associated motoneuron soma size in zebra finches (Wade et al., 2002). Interestingly, E appears to have an active role in the feminization of some dimorphic neuromuscular systems as it decreases syrinx weight and muscle fiber size in juvenile zebra finches (Wade et al., 2002) and feminizes the neuromuscular junction (by increasing synaptic strength) in juvenile African clawed frogs (Tobias and Kelley, 1995).

Androgens and estrogens often also influence sex differences in both the structure and function of these systems in adulthood. This is particularly evident in the rodent copulatory system where treatment with testosterone (T) or dihydrotestosterone (DHT) typically increases the size of both muscles and motoneurons and is required for the expression of the associated behavior

(penile reflexes) (e.g., Hart, 1967; Breedlove and Arnold, 1981; Rand and Breedlove, 1991). However, in adulthood, E does not alter the size of these copulatory muscle fibers (Fargo et al., 2003) or motoneurons (Forger et al., 1992), although it does increase excitability of the muscles (Fargo et al., 2003). In adult African clawed frogs, prolonged T-treatment induces complete masculinization of laryngeal muscles in females (Tobias et al., 1991), and adult ovariectomy decreases quantal content at the synapse (Tobias et al., 1998). T also masculinizes the syrinx muscles, but not associated motoneurons, in adult female zebra finches (Wade and Buhlman, 2000).

Similar to other reproductive neuromuscular systems, the components that underlie copulation in lizards are also sexually dimorphic. Adult male green anoles possess two intromittant copulatory organs called hemipenes. Each hemipenis is independently controlled by two muscles, the transversus penis (TPN; everts the hemipenis) and the retractor penis magnus (RPM; retracts the hemipenis) (Arnold, 1984). These muscles receive projections from ipsilateral populations of motoneurons found in spinal segments trunk 17 and sacral 1 (T17-S1) (Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1). As in many others, androgens have stimulating effects on this neuromuscular system; T-treatment increases size of hemipenes and copulatory muscles (but not motoneurons) in adult male green anoles (Holmes and Wade, 2004b; Chapter 2) and castration decreases these measures (only statistically significant for hemipenes) in juvenile males (Lovern et al., 2004b; Chapter 4). In contrast, E can induce atrophy of hemipenes and copulatory muscles in adult males (Holmes

and Wade, unpublished observations). Adult females do not possess hemipenes or the associated muscles and have fewer and smaller T17-S1 motoneurons (Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1). Juvenile females also do not possess male-typical copulatory neuromuscular structures, and treatment with T at that age does not cause them to develop (Lovern et al., 2004b; Chapter 4), consistent with the idea that sexual differentiation of this system is not reversible once it occurs.

It is clear that the adult lizard copulatory neuromuscular system exhibits responsiveness to steroid hormones similar to other species described above. However, while significant work has focused on fish, frogs, birds, and mammals, little is known about the effects that these hormones have during sexual differentiation of reptilian neuromuscular systems. Androgen treatment has masculinized the hemipenes in embryos of some lizard species and estrogens can inhibit the development of these structures (reviewed in Raynaud and Pieau, 1985), but this work has been done in few species (not the green anole) and the role of these hormones in the differentiation of the underlying muscles and motoneurons was unknown. To address this issue in particular, in Experiment 1 we characterized the time course of normal ontogeny of copulatory neuromuscular morphology in green anoles by collecting embryos at a variety of developmental stages. Using the developmental time points determined, in Experiment 2 we treated embryos with T, DHT, E or vehicle control after gonadal differentiation but prior to differentiation of secondary copulatory structures.

Animals were then collected on the day of hatching, allowing us to determine the effects of hormone treatment on morphology of the neuromuscular components.

MATERIALS AND METHODS

Animals and housing

All embryos and hatchlings came from our breeding colony. Adult male and female green anoles were purchased from Charles Sullivan Co (Nashville, TN). Animals were group housed (1 male with 3-6 females) in 110-liter glass aquaria (76 x 30 x 48 cm) that each contained a nest box (0.5 to 1.0 L plastic containers with one hole cut in the top, 2/3 full of peat moss dampened with distilled water). Lizards were housed in a 14:10 hour light:dark cycle with fluorescent and full-spectrum lights, as well as a heat lamp placed on one end of each aquarium. Temperature ranged from 28°C (ambient) to 38°C (directly under heat lamp) during the day to 18°C at night. These conditions approximate those the animals experience in the spring in the field and stimulate breeding in the lab. Aquaria were sprayed daily to help maintain 70% relative humidity, and water was provided *ad libitum*. Animals were fed crickets or mealworms three times a week. Nest boxes were checked daily for eggs, which were then placed individually in plastic cups containing a 1:1 (mass) vermiculite:dH2O mixture and sealed with plastic wrap. Eggs were incubated at 28°C and hatched approximately 34 days after laying. All procedures were approved by the Michigan State University All University Committee on Animal Use and Care and conform to NIH guidelines.

Treatment, tissue collection, and analyses

Experiment 1. To determine the time course of differentiation of copulatory neuromuscular structures, embryos were collected at one of several points during incubation: days 10, 13, 16, 19, 22, 25, 28, or 31 or on the day of hatching (day 0 was the day of laying). The number of eggs collected at each stage ranged from 5 to 19. Eggs were opened and embryos were removed, weighed and decapitated. Bodies were placed in Bouin's fixative for seven days. Tissue was then soaked in an ascending series of alcohols and xylene, embedded in paraffin, and sectioned rostral to caudal through the abdomen, pelvis and tail at 10 μm. All tissue was stained with hematoxylin and eosin. For each animal, tissue was analyzed for a) classification of gonad (ovary vs. testes), b) presence/absence of hemipenes and associated muscles, and c) renal sex segment cell size (see below). A gonad was classified as an ovary if it had a well-developed cortex with developing follicles and a regressing medulla and as a testis if it contained a well-developed medulla with organized seminiferous tubules with no distinct cortex or follicular cells (see Figure 1). Hemipenes and muscles were identified by their locations on the ventral surface of the tail; they lie immediately inside the skin at the junction of the caudifemoralis (CF), ischiocaudalis (IC) and iliocaudalis (ILC) muscles (see diagram in Ruiz and Wade, 2002). The height of four renal sex segment epithelial cells was measured from each of 4 randomly selected kidney tubules (resulting in 16 measures). The renal sex segments serve as a bioassay for androgen exposure as they function

similar to the mammalian prostate and enlarge in response to these hormones (Cueller et al., 1972; Crews 1980; Winkler and Wade, 1998).

An additional 16 embryos were collected on incubation day 7, 8, or 9 to determine the timing of gonadal differentiation. Tissue was collected and treated as above. Gonads were classified as ovaries or testes as above or as "undifferentiated" if they did not possess distinguishing characteristics (Figure 1).

Motoneurons could not be evaluated in the above tissue as it was stained with hematoxylin and eosin, which does not provide a distinct outline of the somata. Therefore, 11 additional hatchlings (5 female and 6 male) were collected for this purpose. The tissue was collected and processed as above but was stained with thionin. Motoneurons were analyzed as in Lovern et al. (2004b; Chapter 4). To avoid double-counting of cells across sections, motoneuron number was estimated using a modified version of the physical dissector technique (Gunderson, 1986; Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1) in which a cell was counted if it possessed an in-focus nucleolus. Motoneurons were counted in every fifth section throughout the rostro-caudal extent of the T17-S1 nucleus (from the rostral onset of the T17 dorsal nerve to the caudal extent of the S1 dorsal nerve). Counts were multiplied by the sampling ratio, providing an estimate for total motoneuron number for the left and right sides of the spinal cord. Average motoneuron soma size was estimated by measuring the cross-sectional area of 20 motoneurons from each of the left and right sides of the spinal cord in segments T17-S1. A mean was computed for

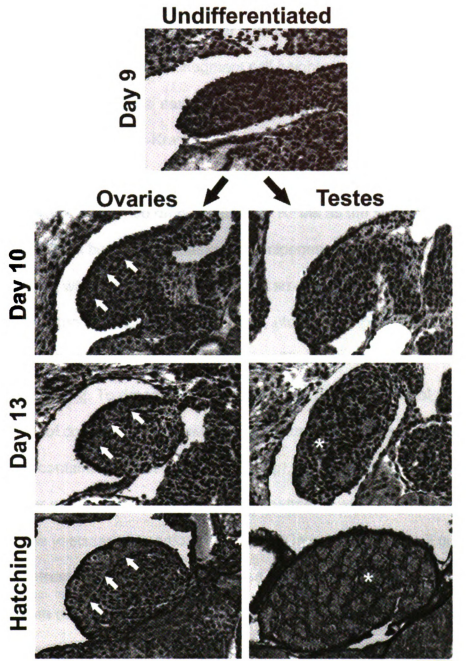

Figure 1. Photomicrographs of cross sections through the gonads of untreated developing green anole embryos. Gonads differentiate around incubation days 9 and 10 (total incubation time is 34 days on average). At days 7-9, gonadal tissue in most individuals did not have defining characteristics of either testes or ovaries. By day 13, ovaries each had a well-developed cortex (noted with white arrows) that contained developing follicles, and testes had well-developed medullas with organized seminiferous tubules (noted with white asterisks). Scale bar = 50 μm.

each side but as there were no effects of laterality (data not shown), analyses below reflect an average of both sides for both motoneuron size and number.

Body weight and renal sex segment cell height were analyzed by two-way ANOVA with age and gonadal sex as independent variables. Posthoc analyses were performed using Tukey-Kramer tests. Motoneuron soma size and number in male and female hatchlings were analyzed with unpaired t-tests. Statistical analyses were not performed on hemipenes or RPMs as the size of these structures could not be reliably measured; hemipenes were everted until shortly before hatching, which makes it difficult to discern the edge of each hemipenis from the wall of the cloaca, and RPMs extend into the hemipenes when everted, which additionally distorts their shape and size (Figure 2).

Experiment 2. To determine whether steroid hormones control differentiation of copulatory neuromuscular structures, eggs were treated with 95% ethanol containing T (100µg), DHT (100µg), E (10µg) or no steroid (number of animals per group is listed in Table 2). All treatments were a 5µl bolus applied topically to the vascularized portion of the shell (Crews et al., 1991). Eggs each received two treatments: one on incubation day 10 and one on incubation day 13. These time points were chosen because gonadal differentiation is complete by day 10 but regression of the copulatory neuromuscular structures is not fully underway (see Results for Experiment 1 below). Eggs remained in the incubator until hatching at which point tissue was collected and processed as for Experiment 1 with one exception. Slides containing the pelvic region of the spinal

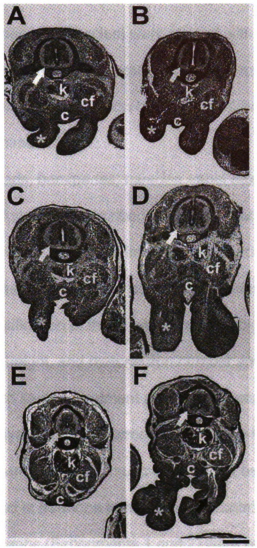

Figure 2. Photomicrographs of cross sections through the pelvic region of untreated developing female (A, C, and E) and male (B, D, and F) embryos. The dorsal surface of each embryo is on the top of each panel and the ventral surface, with hemipenes protruding in most cases, is on the bottom. For orientation, the spinal cords are denoted with white arrows and one hemipenis in each panel except (E) is indicated with a white asterisk. Kidneys (k), cloacal vent (c) and caudifemoralis (cf) muscles are also marked. At incubation day 10 (A and B) both males and females have everted hemipenes. At incubation day 13 (C and D), the hemipenes are decreasing in size in females and increasing in size in males. By incubation day 19 (E and F), hemipenes are absent in females. They remain everted in males at this time. RPM fibers project into the center of the hemipenes when everted and can be seen in (F), indicated with a black arrow. Additional tissue visible in some panels (as in the bottom right corner of (B)) is from the lower limbs of the embryos. Scale bar = 215 μm (A-D), 300 μm (E, F).

cord were stained with thionin while slides containing abdominal tissue (gonads) and tail tissue (hemipenes and muscles) were stained with hematoxylin and eosin. These techniques permitted accurate analyses of motoneuron size and number while also allowing for evaluation of the gonads, hemipenes, and muscles in the same animals.

Analyses of gonads, hemipenes, muscles, renal sex segments, and motoneurons were performed as described for Experiment 1. As genetic sex of the animals could not be determined, animals were classified as males if they possessed testes and as females if they possessed ovaries. There were no cases in which the identification of these organs was ambiguous. Body weight, renal sex segment cell height, and motoneuron number and size were analyzed by two-way ANOVA with steroid treatment and gonadal sex as the independent variables. Posthoc analyses were performed using Tukey-Kramer tests. As in Experiment 1, size of hemipenes and muscles could not be analyzed as hemipenes were everted in hormone treated but not control animals (see below).

All photomicrographs (Experiments 1 and 2) were obtained using an Olympus BX51 microscope with a Macrofire (Optronics) digital camera. All images were sized, compiled, and labeled in Adobe PhotoShop 7.0 (San Jose, CA). In addition, brightness and contrast were adjusted as necessary and retouching was performed to remove background artifacts; no modifications were made to images of the tissue.

RESULTS

Experiment 1. Gonadal differentiation occurred around incubation days 9 and 10 (Figure 1). Gonads were undifferentiated (lacked clear cortex and/or organized seminiferous tubules) in all day 7 (n=5) and day 8 (n=4) embryos and in 4 of 7 day 9 embryos; 3 of 7 day 9 embryos had begun to develop ovarian cortices. By day 10, only 2 of 7 embryos had undifferentiated gonads; of the others, 4 had ovaries and 1 had testes. All animals possessed Wolffian ducts while only animals with ovaries possessed oviducts. All animals possessed hemipenes and rudimentary RPM fibers on incubation day 10 (Figure 2). Hemipenes were everted prior to hatching and increased in size throughout development in gonadal males (Figure 2). RPM fibers organized into distinct, ensheathed muscles by incubation day 19, but may not have been functional until shortly before hatching as that is when hemipenes were retracted (Figure 3). Hemipenes and muscles degenerated in concert in females; they were clearly regressing in all day 13 gonadal females (n=6) and were completely absent in day 19 females (n=5) (Figure 2). Cells in spinal segments T17-S1 differentiated into recognizable multipolar motoneurons between days 10 and 19 and were clearly organized in the ventral horn by days 19-22 (Figure 4). In hatchlings, T17-S1 motoneurons were not sexually dimorphic in size ($t = 0.054$, $p = 0.958$) or number ($t = 0.934$, $p = 0.375$) (Table 1).

Renal sex segment cells were larger in males (overall average = 12.822 +/- 0.204 μm) than females (overall average = 11.241 +/- 0.239 μm) ($F = 18.317$, $p < 0.0001$), but age did not affect this measure ($F = 1.175$, $p = 0.323$). A

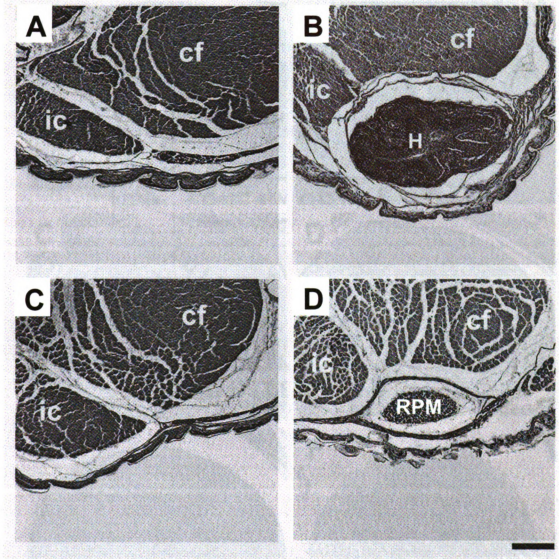

Figure 3. Photomicrographs of cross sections through the tail of two untreated individuals, a female (A and C) and a male (B and D) hatchling. (A) and (B) are at the level of the hemipenes, which are located on the ventral surface of the tail at the junction of the caudifemoralis (cf) and ischiocaudalis (ic) muscles. (C) and (D) are at the level of the RPM, which attaches to the base of the hemipenis in males and is more caudal. Note that hemipenis and RPM are completely absent in the female hatchling. H = hemipenis; RPM = retractor penis magnus. Scale bar = 100 µm.

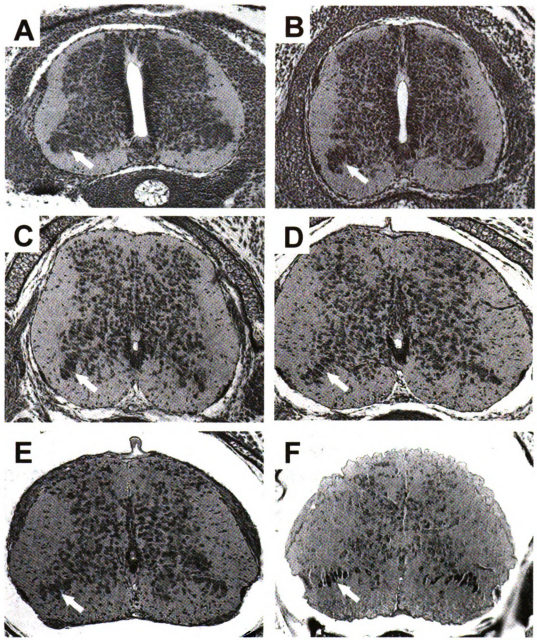

Figure 4. Photomicrographs of cross sections through spinal segment trunk 17 in untreated male embryos on incubation days 10 (A), 13 (B), 19 (C), 22 (D), 25 (E), and at hatching (F). White arrows point to motoneurons in the ventral horn, some of which project to the RPM and TPN muscles. Note the distinct configuration of the motoneurons that becomes apparent around incubation days 19 to 22. These cells are not sexually dimorphic in size or number at the time of hatching. Scale bar = 100 µm.

	T17-S1 count	T17-S1 soma size
Female (n=5)	224.20 (11.20)	149.35 (14.39)
Male (n=6)	249.17 (22.40)	150.41 (12.15)

Table 1. Mean (+/- SEM) motoneuron count and soma size (μm^2) in spinal segments trunk 17 and sacral 1 (T17-S1). Approximately half of the cells in this region project to muscles controlling the hemipenes in adult male green anoles.

statistically significant interaction between gonadal sex and age was detected ($F = 2.715$, $p = 0.010$) as the sex difference appeared only after incubation day 16. Embryos increased in weight as they aged ($F = 64.877$, $p < 0.0001$) from 75.0 (+/-2.35) g on day 10 to 274.86 (+/- 7.25) g on the day of hatching, but this measure did not differ between the sexes ($F = 0.014$, $p = 0.9077$). The interaction between age and sex also was not statistically significant ($F = 0.554$, $p = 0.791$).

Experiment 2. All control animals (8 males and 7 females) had copulatory morphology consistent with their gonadal sex. Only animals with testes possessed hemipenes and associated muscles (Figure 5). These structures were absent in all animals treated with E (14 gonadal females and 4 gonadal males) (Figure 5). All androgen-treated males had hemipenes, RPMs, and TPNs (11 for DHT and 5 for T), and most androgen-treated females also had hemipenes and associated muscles (9 of 10 for DHT and 8 of 10 for T) (Figure 5). Similar to the untreated hatchlings in Experiment 1, T17-S1 motoneurons were not sexually dimorphic in size ($F = 0.852$, $p = 0.360$) or number ($F = 2.060$, $p = 0.157$) in this experiment (Table 2). There was a main effect of steroid treatment on motoneuron size ($F = 2.823$, $p = 0.047$) and number ($F = 2.918$, $p = 0.042$), and a significant interaction between gonadal sex and steroid treatment was detected for motoneuron number ($F = 3.476$, $p = 0.022$) but not for motoneuron size ($F = 0.782$, $p = 0.509$). However, no statistically significant pairwise comparisons between treatment groups were detected on either measure. There was a main effect of steroid treatment on renal sex segment cell height ($F = 16.825$, $p < 0.0001$), but no significant main effect of sex was detected ($F = 0.662$, $p = 0.419$)

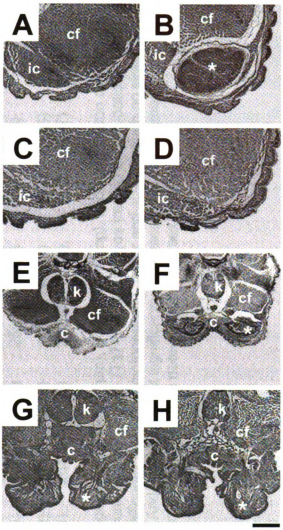

Figure 5. Photomicrographs of cross sections through the tail (A-D) and pelvis (E-H) of treated hatchlings. (A) and (B) are from a control female and male and (C) and (D) are from an E-treated female and male, respectively. (A-D) are at the level of the hemipenes, which are located on the ventral surface of the tail at the junction of the caudifemoralis (cf) and ischiocaudalis (ic) muscles. Note that the hemipenis (marked with asterisks in which they are depicted) is completely absent in the control female as well as both E treated animals. (E) and (F) are the same animals as in (A) and (B) while (G) and (H) are from a DHT treated female and male, respectively. For (E-H) the dorsal surface of each embryo is on the top of each panel and the ventral surface is on the bottom. For orientation, kidneys (k), cloacal vent (c) and cf muscles are noted. Note that hemipenes are absent in the control female and are everted in both the DHT treated female and male. The retracted hemipenes in (B) and (F) appear different because they are at different levels; (B) is approximately midway through the rostral-caudal extent of the hemipenis while (F) is at the level of the cloacal vent, right at the top of the hemipenes. Scale bar = 150 µm (A-D) and 300 µm (E-H).

Treatment	Sex (n)	RSS	Body weight	T17-S1 count	T17-S1 size
Control	Female (7)	10.3 (0.84)	255.29 (15.32)	173.21 (19.29)	125.79 (7.14)
	Male (8)	12.8 (0.55)	259.11 (16.72)	208.75 (12.03)	127.49 (4.40)
E	Female (14)	14.8 (0.40)	250.43 (10.03)	223.46 (13.89)	145.72 (5.30)
	Male (4)	12.7 (1.05)	255.50 (23.17)	160.00 (5.20)	139.03 (10.06)
T	Female (10)	16.3 (1.09)	228.80 (14.42)	213.75 (13.79)	138.49 (6.29)
	Male (7)	18.0 (1.64)	270.29 (10.20)	259.64 (31.89)	148.03 (4.5)
DHT	Female (10)	18.0 (1.32)	253.90 (10.74)	191.56 (16.92)	128.37 (5.05)
	Male (11)	18.6 (1.34)	264.46 (14.04)	250.46 (13.16)	141.05 (6.07)

Table 2. Mean (+/- SEM) renal sex segment cell heights (μm), body weight (mg), and motoneuron count and soma size (μm^2) in spinal segments trunk 17 and sacral 1 (T17-S1) for embryos treated on incubation days 10 and 13. Overall, steroid treatment significantly increased motoneuron number and size although no significant pairwise comparisons were detected. Androgen treatment significantly increased renal sex segment cell height in both males and females. No differences in weight were detected for either sex or treatment.

(Table 2). The interaction between treatment and gonadal sex was not statistically significant ($F = 1.424$, $p = 0.244$). Post hoc comparisons demonstrate that T- and DHT-treated animals, regardless of gonadal sex, had larger cells than control animals (both $p < 0.05$). Neither steroid treatment ($F = 0.191$, $p = 0.902$) nor gonadal sex ($F = 2.143$, $p = 0.148$) nor the interaction between these variables ($F = 0.734$, $p = 0.535$) affected body weight (Table 2).

DISCUSSION

Summary

Differentiation of the copulatory neuromuscular system in green anoles occurs during embryonic development but after gonadal differentiation, which is largely complete by incubation day 10 (total incubation time at 28°C is 34 days on average). Both male and female embryos possess hemipenes and the associated musculature early in development, but these structures completely regress in females by incubation day 19. The motoneurons projecting to copulatory muscles are not sexually dimorphic in size or number in hatchlings. As these cells are both larger and more numerous in adult males than females, this result suggests a dissociation in the time course of differentiation in central versus peripheral neuromuscular components in this system. Treating embryos with androgens (either T or DHT) rescued the hemipenes and muscles in female embryos, whereas treatment with E caused the system to regress in males, suggesting that both types of steroid may be actively involved in sexual differentiation in this system. The effects of androgenic and estrogenic hormones

on hemipenis development in green anoles are generally consistent with those reported for the few other lizard species investigated (reviewed in Raynaud and Pieau, 1985). In addition, the present work demonstrates that the associated muscles are also responsive to both classes of steroid. Overall, steroid treatment increased the size and number of associated motoneurons, although as no significant pairwise comparisons were detected it is difficult to determine the roles of the specific hormones. In sum, embryonic androgens may masculinize and estrogens may feminize peripheral components of the lizard copulatory neuromuscular system, while other mechanisms (or the same mechanisms at a different time point) likely regulate differentiation of the associated motoneurons.

Dissociation between neural and muscular components

The timing of differentiation of neuromuscular components differs across species. For example, dimorphisms in the central nervous system precede differentiation of the muscles in the vocal neuromuscular systems of plainfin midshipman fish (Brantley et al., 1993a; Knapp et al., 1999) and African clawed frogs (Kelley and Dennison, 1990). In contrast, in the rodent copulatory system, dimorphisms in the muscles develop first and mediate differentiation of the associated motoneurons likely via release of one or more trophic factors (reviewed in Breedlove et al., 2002). The present data suggest that differentiation of the lizard copulatory system is in some ways similar to that in rodents as the hemipenes and RPM and TPN muscles clearly differentiate prior to the motoneurons. However, a particularly intriguing aspect of the present data is the

large dissociation in timing between differentiation of the motoneurons compared to the muscles and target structures (hemipenes), which is not seen in other species. While the motoneurons are clearly organized in the ventral horn by the time of hatching, they are not sexually dimorphic in size or number; males had more cells than females in Experiment 1 and in three of four groups in Experiment 2 but the difference was not statistically significant. This lack of a sex difference in cell number exists despite a complete regression of the target musculature in females more than 15 days earlier (Figure 3). Even more striking is the fact that these motoneurons are still not dimorphic in size or number in juvenile anoles at 3 months after hatching (Lovern et al., 2004b; Chapter 4). Comparing cell counts in hatchling (present data) or juvenile (Lovern et al., 2004b; Chapter 4) with adult anoles (Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1) suggests that the sex difference in cell number may arise from a decrease in motoneurons in adult females rather than motoneuron genesis in adult males. One hypothesis is that the motoneurons differentiate as the animals enter their first breeding season, the first time point at which males exhibit adult levels of T (Lovern et al., 2001). However, the motoneurons do not appear to be sensitive to steroid hormones in later adulthood (at least in regards to soma size; Holmes and Wade, 2004b; Chapter 2), so an androgenic mechanism may not be involved. Another possibility is that differentiation of the cells is influenced by sex differences in use that occur once the animals reach reproductive maturity. Thus, the motoneurons may exhibit an as yet undefined "pubertal" period of sensitivity

to T at the beginning of reproductive maturity, or differentiation may be mediated by a non-steroidal mechanism.

Steroid hormone effects on copulatory neuromuscular structures

Differentiation of sexually dimorphic neuromuscular systems is typically mediated by gonadal steroids. Juvenile androgens masculinize components of the vocal motor systems of African clawed frogs (Sassoon and Kelley, 1986; Robertson et al., 1994; Kay et al., 1999), plainfin midshipman fish (Brantley et al., 1993b), and possibly zebra finches, although exogenous estrogens also feminize other components in this species (Wade et al., 2002; see below). Perhaps the closest comparison to the present data, however, is the rodent copulatory neuromuscular system. Here the neural and muscular tissues develop in both sexes but regress in females shortly after birth; androgens clearly mediate differentiation of the muscles, which in turn control survival of the motoneurons (e.g., Breedlove et al., 1982; Breedlove and Arnold, 1983a, 1983b; Nordeen et al., 1985). Differentiation of the green anole copulatory neuromuscular system parallels this pattern in that hemipenes, RPMs, TPNs, and associated motoneurons are present in both sexes embryonically, and the peripheral components regress early in development in females. As treatment with androgens can in both cases rescue these structures in females, it is clear that a masculinizing role for androgens appears to be conserved.

Of particular interest, though, is the active feminization induced by E in anoles that appears less conserved across vertebrates. E can play a role in the

feminization of the sexually dimorphic passerine song system as it decreases syrinx weight and muscle fiber size in juvenile zebra finches (Wade et al., 2002). In contrast, E has masculinizing properties in the developing rodent copulatory system as it increases both the soma size (Breedlove, 1997b) and dendritic outgrowth (Goldstein and Sengelaub, 1994) of motoneurons. It will be important in the future to solidify the role of exogenous androgens and estrogens in anoles by inhibiting exposure to these hormones during embryonic development.

It is also worth exhibiting some caution in interpreting the results for another reason. In contrast to mammals, steroid hormones strongly influence differentiation of both avian and reptilian gonads. Inhibiting E synthesis induces testicular development in female birds (Elbrecht and Smith, 1992; Wade and Arnold, 1996) while E treatment disrupts testicular morphology in males (Wade et al., 1997). Similarly, treating reptilian eggs with E causes embryos to develop ovaries in some species (Bull et al., 1988; Crews et al., 1991; Wibbels and Crews, 1992; Tousignant and Crews, 1994; Wibbels and Crews, 1995), whereas T induces testicular development in at least one other (Ganesh and Raman, 1995). As there is no method at present to determine the genetic sex of green anoles, we cannot be sure that the gonads were not affected in our steroid-treated animals. In particular, one might be concerned that the low number animals with testes following E treatment (4 of 18) is a result of E feminizing the gonads. However, it seems unlikely that our treatments altered gonadal development as they commenced after the time point of differentiation and we saw no ambiguity in their structure in Experiment 2. More likely is our intended

scenario of the lipophillic steroids providing exogenous hormone exposure to the animal until hatching, probably following absorption into the yolk. Consistent with this idea is that androgen treated animals had larger renal sex segment cells (indicates androgenic activity) at the time of hatching – two weeks after the second (and final) steroid treatment. An additional possibility is that steroid treatment might have altered production of endogenous gonadal hormones, resulting in an indirect effect on copulatory neuromuscular morphology.

It has been hypothesized that mechanisms of sex determination may dictate which sex is "default", that the homogametic sex is neutral and easier to sex reverse whereas the heterogametic sex possesses the dominant gonad that produces hormones controlling differentiation (Adkins-Regan, 1981). The present data challenge these ideas. While estrogenic steroids actively feminize genitalia in *Lacerta viridis*, a lizard with female heterogametic sex determination (ZZ/ZW, as in birds), E has the same effect in green anoles, which, like mammals, exhibit male heterogametic sex determination (XX/XY, as in mammals) (Adkins-Regan, 1981). As such, it may be that the role of steroid hormones in sexual differentiation is not determined by mechanism of sex determination *per se*. Indeed, the present data suggest that androgenic and estrogenic hormones both actively contribute to the differentiation of male and female green anoles, implying that neither may be "default" in this species.

DISCUSSION

Species used in the study of sexually dimorphic neuromuscular systems involved in reproduction typically are useful for examining either courtship systems (e.g., in fishes, frogs, and birds; see Chapter 1) or copulatory systems (e.g., in mammals; see Chapter 1), but generally not both. Many non-mammalian species do not possess intromittant organs for reproduction, and most mammals do not exhibit courtship behaviors that are easily measurable in the laboratory. Thus, green anoles provide the opportunity to simultaneously examine mechanisms regulating two neuromuscular systems required for the full suite of masculine reproductive behaviors: one controlling the use of their copulatory organs and another controlling extension of a throat fan (dewlap) used in courtship. The present work largely focused on the green anole copulatory neuromuscular system because it exhibits a remarkable degree of sexual dimorphism. Adult males possess two hemipenes and their associated muscles and motoneurons while adult females do not (Ruiz & Wade, 2002; Holmes and Wade, 2004a; Chapter 1). Given its critical role in male reproduction, it is not surprising that components of this system are responsive to testosterone (T), the most potent steroid activator of male-typical reproductive behaviors in this species (Mason & Adkins, 1976; Crews et al., 1978; Adkins & Schlesinger, 1979; Winkler & Wade, 1998). What is surprising, however, is the pattern of responsiveness that these structures exhibit. The results presented in the

preceding chapters clearly demonstrate several conclusions about T action on the anole copulatory system.

1) Seasonally-induced morphological plasticity is limited

Precedence for naturally occurring neural and muscular plasticity exists in seasonally breeding species. For example, the song control system in the passerine brain exhibits remarkable structural and functional plasticity (Brenowitz, 2004; Park et al., 2005) associated with periods of song learning. Similarly, the copulatory neuromuscular system of seasonally breeding rodents changes in parallel with reproductive behavior. Motoneurons in the spinal nucleus of the bulbocavernosus and their associated muscles are larger in males housed in breeding than non-breeding environmental conditions (Forger and Breedlove, 1987; Hegstrom and Breedlove, 1999). The effects of season are typically due to direct activity of T; levels of the hormone increase corresponding to, or in anticipation of, breeding conditions. However, photoperiod can also induce plasticity in some neuromuscular measures independent of androgens (e.g., neuromuscular junction size; Hegstrom et al., 2002).

Green anoles are strict seasonal breeders, exhibiting courtship and copulatory behaviors only when days are long and temperatures are warm (Jenssen et al., 1995; 1996). T levels rise and fall in parallel with reproduction and are approximately twice as high during the breeding than non-breeding season (Lovern et al., 2001). The muscles and motoneurons that control dewlap extension do not appear to respond morphologically to changes in environmental

conditions (O'Bryant and Wade, 1999). However, as both males and females occasionally use their dewlaps in the non-breeding season, it is possible that a lack of seasonal effect on morphology is due to required maintenance of the system. Comparing copulatory neuromuscular morphology in gonadally intact male anoles housed in either breeding or non-breeding environmental conditions (Holmes and Wade, 2004b; Chapter 2) demonstrated that seasonal cues also do not induce morphological changes in this system. The only measure that differed between seasons in gonadally intact animals was a very small change in motoneuron soma size, which was not limited to copulatory motoneurons. One possibility is that copulatory neuromuscular morphology is insensitive to T and/or other seasonal cues. More likely, however, is that endogenous fluctuations in T are not robust enough to induce morphological change. Although the copulatory system may not exhibit remarkable naturally occurring fluctuations in morphology, it does have the capacity for plasticity. Indeed, seasonal cues have indirect effects on copulatory neuromuscular morphology by mediating the responsiveness of these structures to exogenous T (see below).

2) Exogenous T has trophic effects on the periphery in adulthood

While T is the most potent steroid for the activation of both courtship and copulatory behaviors in adult green anoles (Mason and Adkins, 1976; Crews et al., 1978; Adkins and Schlesinger, 1979; Winkler and Wade, 1998), it does not induce morphological change in the adult dewlap neuromuscular system. Dewlap motoneurons and CH muscle fibers do not appear to differ between castrated

and T-treated males (O'Bryant and Wade, 1999). Yet T-treatment increases RPM muscle fiber size and hemipenis size in the copulatory system. Interestingly, this effect is only seen when animals are housed in breeding environmental conditions (Holmes and Wade, 2004b; Chapter 2), suggesting that seasonal cues can influence responsiveness to exogenous T.

The trophic effect of T on the muscles and hemipenes of the copulatory system is consistent with other species; exogenous T commonly influences peripheral components in sexually dimorphic neuromuscular systems of a wide variety of vertebrates. For example, adult T-treatment increases muscle fiber size in the syrinx of female zebra finches (Wade and Buhlman, 2000). Similarly, androgens increase the mass of external oblique muscles of gray tree frogs (used in sound production; Girgenrath and Marsh, 2003) and sonic muscles in Type II male plainfin midshipman fish (Lee and Bass, 2005). Testicular implants increase muscle fiber number (Watson et al., 1993) and long term T-treatment masculinizes muscle fiber type (Sassoon et al., 1987; Tobias et al., 1991) in the larynx of adult female Xenopus. Finally, T increases fiber size (Rand and Breedlove, 1991) and twitch capacity (Souccar et al.,1992) in the muscles of the adult rodent copulatory system.

Given that exogenous T, but not normally occurring T fluctuation, alters copulatory morphology, it is important to address how these data are relevant to the reproductive function of green anoles. The difference in circulating T between castrated males and those that received T implants in the studies in this dissertation was substantially greater than the difference between intact males in

the simulated breeding and non-breeding seasons. These two approaches, of comparing gonadally intact males as well as castrated males with and without T-treatment, allows us to explore both what naturally occurs in these animals, and importantly, what the possibilities or limits are for the system. In regards to the copulatory system, the data suggest that sufficient T is present in the non-breeding season to maintain copulatory neuromuscular morphology in gonadally intact males. Alternatively, decreased responsiveness to T in non-breeding conditions, as demonstrated in castrated males treated with T, may serve to protect copulatory structures during periods of low circulating hormones.

3) Morphology of copulatory muscles, but not the motoneurons, is responsive to T

The data presented above demonstrate a dissociation between T responsiveness in the periphery of the copulatory system versus the central nervous system. While the hemipenes and muscles appear sensitive to exogenous T in adulthood, the corresponding T17-S1 motoneurons are not, at least in terms of soma size (Holmes and Wade, 2004b; Chapter 2). This insensitivity is consistent with the dewlap motoneurons in green anoles, which also fail to exhibit changes in soma size following androgen manipulation (O'Bryant and Wade, 1999), as well as the syrinx motoneurons in the zebra finch song system (Wade and Buhlman, 2000) and sonic motor nucleus in Type II male plainfin midshipman fish (Lee and Bass, 2005). In contrast, motoneuron soma size in the adult female canary song system increases in response to T-

treatment (DeVoogd et al., 1991), as do motoneurons in the rodent copulatory system (Breedlove and Arnold, 1981). Taken together, responsiveness in the muscles and/or target tissues does not necessarily parallel sensitivity in the corresponding motoneurons. Of course, there are many additional morphological and functional parameters that T could be manipulating. As such, a true dissociation between motoneuron and muscle sensitivity has not yet been determined.

4) T may have its morphological effects via upregulation of AR

T increases AR expression in the anole copulatory neuromuscular system in a manner parallel to its effects on morphology, whereby the percent of AR+ nuclei is greater in RPM muscle fibers following T-treatment (Holmes and Wade, submitted; Chapter 3). Interestingly, T increased AR expression in the RPMs of males housed in either breeding or non-breeding conditions, although morphological effects are only seen during the breeding season. While AR is expressed in a high percentage of cells that do not exhibit morphological responsiveness to T, including dewlap muscle fibers (approximately 60-70%) and copulatory motoneurons (approximately 70-80%), T-treatment did not alter the percentage of these cells that were AR+ (Holmes and Wade, submitted; Chapter 3). Furthermore, treatment with estradiol causes atrophy of the hemipenes and muscles in adulthood (Holmes and Wade, unpublished observations), demonstrating that the trophic effects of T are not a result of aromatization to estradiol. As AR expression is relatively high in structures that do not respond

morphologically to T, the data also demonstrate that presence of AR does not alone confer morphological responsiveness to T, although it is critical to acknowledge that additional morphological and/or physiological parameters that we have not yet measured may be sensitive to this hormone. This dissociation between AR expression and morphological responsiveness to testosterone is similar to the dorsolateral nucleus of the rat copulatory neuromuscular system. Approximately 90% of the motoneurons projecting to the external urethral sphincter are AR+ (Jordan, 1997), but these cells to not change in size following T manipulation (Collins et al., 1992).

These types of data have interesting implications for potential sites of action of this hormone. Does T act directly on the muscle to increase fiber size or does the trophic response occur indirectly due to increased hemipenis size? Although unlikely, T could also be acting on AR in the copulatory motoneurons and having an anterograde effect on muscles and hemipenes. Given the increased expression of AR in RPM muscle fibers following T-treatment (Holmes and Wade, submitted; Chapter 3), as well as the high expression in the hemipenes, it is likely that testosterone can act directly on both muscles and hemipenes, causing their respective sizes to change in concert with each other. It is likely that a minimum hemipenis size is required for male reproductive success and it is possible, although purely speculative, that increased hemipenis size increases fertility (perhaps facilitating sperm transfer, sperm competition, etc). Perhaps larger hemipenes would need larger muscles to maneuver them, explaining why the effects of T are typically parallel in both structures. But why is

there no effect of T on the size of the corresponding motoneurons? The functional significance of the relationship between motoneuron and muscle fiber size in this system is less clear. While muscle and motoneuron are typically size-matched (reviewed in Gordon et al., 2004) and respond in parallel to T (reviewed in Breedlove et al., 2002) in mammalian neuromuscular systems, it is not apparent that a similar relationship exists in the lizard copulatory neuromuscular system.

5) Testosterone has trophic effects on periphery during juvenile development

The dewlap neuromuscular system begins to differentiate in juveniles between 30 and 90 days post-hatching. The second ceratobranchial cartilages and CH muscles become larger in males than females during this period (O'Bryant and Wade, 2001). Treating juvenile females with T increases cartilage length and CH muscle fiber size, demonstrating that the dewlap system is sensitive to T during development (Lovern et al., 2004b; Chapter 4). In contrast, hemipenes or RPM muscle fibers were not evident in these females, suggesting that sexual differentiation of the copulatory neuromuscular system is complete by this time, and is not reversible by T-treatment once finished (Lovern et al., 2004b; Chapter 4). Paralleling T-manipulation in adulthood, castrating juvenile males decreased hemipenis size, and to a lesser extent, RPM fiber size. Both courtship and copulatory motoneurons were not sexually dimorphic in size and number on the day of hatching, and neither population responded to T-manipulation (Lovern et al., 2004b; Chapter 4). Therefore, the dissociation between T sensitivity in

peripheral copulatory structures and their associated motoneurons is also present in juveniles. Furthermore, a dissociation between the timing of sexual differentiation in the dewlap versus copulatory systems clearly exists. While periphery of copulatory system is sensitive to T manipulation in juveniles, differentiation of these structures is complete by day 90 post-hatching, illustrating that the time point of differentiation in this system is much earlier than the dewlap system (see below).

Juvenile development is also a period for steroid sensitivity in other species. T and 11-ketotestosterone increase sonic muscle mass and fiber phenotype in juvenile male and female plainfin midshipman fish, as well as muscle fiber number in males only (Brantley et al., 1993b). T masculinizes muscle fiber type and size in the larynx of juvenile *Xenopus* (Sassoon et al., 1987) and DHT masculinizes motoneuron number in female *Xenopus* tadpoles (Kay et al., 1999). Post-hatching DHT-treatment increases nXIIts motoneuron soma size and overall syrinx weight in female zebra finches, and E decreases syrinx weight and muscle fiber size in juvenile males (Wade et al., 2002). Finally, peripubertal steroids influence motoneuron morphology in the rodent copulatory neuromuscular system. T, DHT, and E increase dendritic length in juvenile male rats, but only T and DHT increase motoneuron soma size (Goldstein and Sengelaub, 1990; Goldstein and Sengelaub, 1994). Interestingly, T may increase motoneuron number in the copulatory system of pubertal male Mongolian gerbils (Siegford and Ulibarri, 2004).

6) Testosterone has trophic effects on periphery during embryonic development

The present data demonstrate that the hemipenes and associated muscles are present in both sexes during early embryonic development but completely regress in female embryos by the time of hatching (Holmes and Wade, in press; Chapter 5). They also suggest that androgenic mechanisms are likely involved to some extent in the differentiation of this system. Treating embryos with androgens (either T or DHT) rescued the hemipenes and associated muscles in almost all embryos (Holmes and Wade, in press; Chapter 5). Androgens also mediate differentiation of sexually dimorphic neuromuscular systems in other species, although differences in the details clearly exist. Androgens masculinize components of the vocal motor systems of African clawed frogs (Sassoon and Kelley, 1986; Robertson et al., 1994; Kay et al., 1999), plainfin midshipman fish (Brantley et al., 1993b), and possibly zebra finches, although exogenous estrogens also feminize other components in this species (Wade et al., 2002; see below). In the rodent copulatory neuromuscular system, the neural and muscular tissues develop in both sexes but regress in females shortly after birth; androgens clearly mediate differentiation of the muscles, which in turn control survival of the motoneurons (e.g., Breedlove et al., 1982; Breedlove and Arnold, 1983a, 1983b; Nordeen et al., 1985). As such, differentiation of the green anole copulatory neuromuscular system most closely parallels the mammalian pattern in that hemipenes, RPMs, TPNs, and associated motoneurons are present in both sexes embryonically, and the peripheral components regress early in development in females (Holmes and

Wade, in press; Chapter 5). As treatment with androgens can rescue these structures in females, it is clear that a masculinizing role for androgens in peripheral structures is somewhat conserved across species.

There may also be a role for estrogens in differentiation of the anole copulatory system as treating embryos with estradiol caused a regression of hemipenes and associated muscles (Holmes and Wade, in press; Chapter 5). This is of particular interest as the active feminization by E appears less conserved across vertebrates. E can play a role in the feminization of the sexually dimorphic passerine song system as it decreases syrinx weight and muscle fiber size in juvenile zebra finches (Wade et al., 2002). In contrast, E has masculinizing properties in the developing rodent copulatory system as it increases both the soma size (Breedlove, 1997b) and dendritic outgrowth (Goldstein and Sengelaub, 1994) of motoneurons. It will be important in the future to better characterize the role of exogenous androgens and estrogens in anoles by inhibiting exposure to these hormones during embryonic development.

The time point of differentiation of the copulatory motoneurons, and the mechanism(s), has not yet been identified. They have not differentiated by the time of hatching (Holmes and Wade, in press; Chapter 5) or by 90 days post-hatching (Lovern et al., 2004b; Chapter 4), but are clearly sexually dimorphic in both size and number in adults (Ruiz and Wade, 2002; Holmes and Wade, 2004a; Chapter 1). These cells also fail to respond to hormone manipulation at all life stages that have been investigated. The estimated number of motoneurons at both developmental time points demonstrates that the masculine

number of cells is present in both sexes, suggesting that differentiation of motoneuron number is due to a loss of cells (or cell death) in females rather than an increase in cells (motoneuron genesis) in males. If this is indeed the case, it is perplexing that these cells are surviving in females at least 3 months after their target muscles have completely regressed.

Taken together, it is clear that peripheral components of the anole copulatory neuromuscular system, the hemipenes and RPM muscle fibers, exhibit trophic responses to T during embryonic (Holmes and Wade, in press; Chapter 5) and juvenile (Lovern et al., 2004b; Chapter 4) development as well as in adulthood (Holmes and Wade, 2004b; Chapter 2). This lifelong sensitivity to testosterone is consistent with the hypothesis that androgens are necessary for both the differentiation and maintenance of these sexually dimorphic structures. The data also suggest that the effects of testosterone are likely due to activation of AR. Further understanding the degree to which components of the system respond to T in regards to additional morphological and functional measures will help to elucidate the extent of this maintenance, as well as shed light on the significance that this T responsiveness has on the reproductive success of male green anoles.

Prior to the work on the green anole, a role for androgens had been demonstrated in sexually dimorphic neuromuscular systems in all vertebrate classes (including fish, frogs, birds, and mammals) except for reptiles. As such, these results, in addition to work demonstrating androgenic mediation of the

leopard gecko copulatory system (Holmes et al., 2005), fill a critical gap in the comparative approach to understanding steroid hormone effects on dimorphic neuromuscular systems.

Comparing across species, some relationship between structure and function clearly exists in all sexually dimorphic neuromuscular systems. But, collectively, the work with the anole has made it clear that this relationship between morphology and behavior is not nearly as simple as it first appears. This idea is illustrated with two points: 1) both the dewlap and copulatory systems experience a huge seasonal variation in use but do not exhibit naturally occurring morphological plasticity and 2) differentiation and adult plasticity in the copulatory muscles does not parallel the corresponding motoneurons. In addition, the present work illustrates selective sensitivity to T within and between neuromuscular systems. Directly comparing the dewlap and copulatory neuromuscular systems is particularly powerful because they are presumably exposed to the same circulating hormones at the same developmental time points. Given that the systems differentiate at independent stages and have dissimilar responses to T in adulthood, it is clear that sensitivity to this hormone is tissue specific and can be turned on and off at different times. This specificity is likely mediated by factors in addition to the presence of AR; this protein is expressed in neuromuscular effectors that respond to T as well as those that do not (Holmes and Wade, submitted; Chapter 3). Candidate mechanisms for conferring selective hormone sensitivity include steroid metabolizing enzymes (e.g. 5-α-reductase and aromatase) as well as steroid hormone receptor co-

activators. Understanding how additional morphological, neurochemical, and electrophysiological parameters are influenced by T in diverse species will help identify these mechanisms and how they are conserved across vertebrates.

LITERATURE CITED

Adkins E, Schlesinger L (1979). Androgens and the social behavior of male and female lizards (*Anolis carolinensis*). Horm Behav 13: 139-152.

Adkins-Regan E (1981). Early organizational effects of hormones: an evolutionary perspective. In: Adler NT, editor. Neuroendocrinology of Reproduction: Physiology and Behavior. New York: Plenum Press. p 159-228.

Andersson M (1994). Sexual Selection. Princeton University Press, Princeton USA.

Akita K (1992). An anatomical investigation of the muscles of the pelvic outlet in iguanas (Iguanidae *Iguana iguana*) and varanus (Varanidae *Varanus* (*dumerillii*)) with special reference to their nerve supply. Ann Anat 174:119-129.

Arnold AP (1975). The effects of castration and androgen replacement on song, courtship, and aggression in zebra finches (*Poephila guttata*). J Exp Zool 191: 309-326.

Arnold AP (1980). Effects of androgens on volumes of sexually dimorphic brain regions in the zebra finch. Brain Res 185: 441-444.

Arnold AP (1997). Sexual differentiation of the zebra finch song system: positive evidence, negative evidence, null hypotheses, and a paradigm shift. J Neurobiol 33: 572-584.

Arnold AP, Breedlove SM (1985). Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm Behav 19: 469-498.

Arnold EN (1984). Variation in the cloacal and hemipenial muscles of lizards and its bearing on their relationships. Symp Zool Soc London 52: 47-85.

Balice-Gordon RJ, Breedlove SM, Bernstein S, Lichtman JW (1990). Neuromuscular junctions shrink and expand as muscle fiber size is manipulated: in vivo observations in the androgen-sensitive bulbocavernosus muscle of mice. J Neurosci 10: 2660-2671.

Bass AH (1985). Sonic motor pathways in teleost fishes: A comparative HRP study. Brain Behav Evol 27:115-131.

Bass AH, Marchaterre MA (1989a). Sound-generating (sonic) motor system in a teleost fish (*Porichthys notatus*): sexual polymorphism in the ultrastructure of myofibrils. J Comp Neurol 286: 141-153.

Bass AH, Marchaterre MA (1989b). Sound-generating (sonic) motor system in a teleost fish (*Porichthys notatus*): sexual polymorphisms and general synaptology of sonic motor nucleus. J Comp Neurol 286: 154-69.

Bass AH, Baker R (1990). Sexual dimorphisms in the vocal control system of a teleost fish: morphology of physiologically identified neurons. J Neurobiol 21: 1155-1168.

Bels VL (1990). The mechanism of dewlap extension in *Anolis carolinensis* (Reptilia: Iguanidae) with histological analysis of the hyoid apparatus. J Morph 206:225-244.

Bertolucci C, Foa A (1998). Seasonality and role of SCN in entrainment of lizard circadian rhythms to daily melatonin injections. Am J Physiol 274: R1004-1014.

Bertolucci C, Foa A, Van't Hof TJ (2002). Seasonal variations in circadian rhythms of plasma melatonin in ruin lizards. Horm Behav 41: 414-419.

Bottjer SW, Glaessner SL, Arnold AP (1985). Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. J Neurosci 5: 1556-1562.

Brantley RK, Bass AH (1994). Alternative male spawning tactics and acoustic signals in the plainfin midshipman fish *Porichthys notatus* Girard (Teleostei, Batracoididae). Ethology 96: 213-232.

Brantley RK, Tseng J, Bass AH (1993a). The ontogeny of inter- and intrasexual vocal muscle dimorphisms in a sound-producing fish. Brain Behav Evol 42: 336-349.

Brantley RK, Marchaterre MA, Bass AH (1993b). Androgen effects on vocal muscle structure in a teleost fish with inter- and intra-sexual dimorphism. J Morph 216: 305-318.

Brantley RK, Wingfield JC, Bass AH (1993c). Sex steroid levels in *Porichthys notatus*, a fish with alternative reproductive tactics, and a review of the hormonal bases for male dimorphism among teleost fishes. Horm Behav 27: 332-347.

Breedlove SM (1997a). Sex on the brain. Nature 389: 801.

Breedlove SM (1997b). Neonatal androgen and estrogen treatments masculinize the size of motoneurons in the rat spinal nucleus of the bulbocavernosus. Cell Mol Neurobiol 17: 687-697.

Breedlove SM, Arnold AP (1980). Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord. Science 210: 564-566.

Breedlove SM, Arnold AP (1981). Sexually dimorphic motor nucleus in the rat lumbar spinal cord: response to adult hormone manipulation, absence in androgen-insensitive rats. Brain Res 225: 297-307.

Breedlove SM, Arnold AP (1983a). Hormonal control of a developing neuromuscular system. I. Complete demasculinization of the male rat spinal nucleus of the bulbocavernosus using the anti-androgen flutamide. J Neurosci 3: 417-423.

Breedlove SM, Arnold AP (1983b). Hormonal control of a developing neuromuscular system. II. Sensitive periods for the androgen-induced masculinization of the rat spinal nucleus of the bulbocavernosus. J Neurosci 3: 424-432.

Breedlove SM, Jacobson CD, Gorski RA, Arnold AP (1982). Masculinization of the female rat spinal cord following a single neonatal injection of testosterone proprionate but not estradiol benzoate. Brain Res 237: 173-181.

Breedlove SM, Cooke BM, Jordan CL (1999). The orthodox view of brain sexual differentiation. Brain Behav Evol 54: 8-14.

Breedlove SM, Jordan CL, Kelley DB (2002). What neuromuscular systems tell us about hormones and behavior. In Hormones, Brain and Behavior, Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, editors. Academic Press: USA.

Brenowitz EA (2004). Plasticity of the adult avian song control system. Ann N Y Acad Sci 1016: 560-585.

Brenowitz EA, Lent K (2002). Act locally and think globally: Intracerebral testosterone implants induce seasonal-like growth of adult avian song control circuits. Proc Natl Acad Sci 99: 12421-12426.

Bull JJ, Gutzke WHN, Crews D (1988). Sex reversal by estradiol in three reptilian orders. Gen Comp Endo 70: 425-428.

Carsia RV, John-Alder J (2003). Seasonal alterations in adrenocortical cell function associated with stress-responsiveness and sex in the eastern fence lizard (*Sceloporus undulatus*). Horm Behav 43: 408-420.

Cihák R, Gutmann E, Hanzlíková V (1970). Involution and hormone-induced persistence of the M. sphincter (levator) ani in female rats. J Anat 106: 93-110.

Clancy B, Cauller LJ (1998). Reduction of background autofluorescence in brain sections following immersion in sodium borohydride. J Neurosci Methods 83: 97-102.

Collins WF, Seymour AW, Klugewicz SW (1992). Differential effects of castration on the somal size of pudendal motoneurons in the adult male rat. Brain Res 577: 326-330.

Cooke B, Hegstrom CD, Villeneuve LS, Breedlove SM (1998). Sexual differentiation of the vertebrate brain: principles and mechanisms. Front Neuroendocrinol 19: 323-362.

Cooke BM, Hegstrom CD, Breedlove SM (2002). Photoperiod-dependent response to androgen in the medial amygdala of the Siberian hamster, *Phodopus sungorus*. J Biol Rhyth 17: 147-154.

Crews D (1978). Hemipenile preference: Stimulus control of male mounting behavior in the lizard *Anolis carolinensis*. Science 199:195-196.

Crews D (1980). Interrelationships among ecological, behavioral, and neuroendocrine processes in the reproductive cycle of *Anolis carolinensis* and other reptiles. In Advances in the study of behavior, Rosenblatt JS et al., editors. Academic press: New York.

Crews D, Bull JJ, Wibbels T (1991). Estrogen and sex reversal in turtles: a dose-dependent phenomenon. Gen Comp Endo 81: 357-364.

Crews D, Traina V, Wetzel FT, Muller C (1978). Hormonal control of male reproductive behavior in the lizard, *Anolis carolinensis*: role of testosterone, dihydrotestosterone, and estradiol. Endocrinology 103: 1814-1821.

Cuellar HS, Roth JJ, Fawcett JD, Jones RE (1972). Evidence for sperm sustenance by secretions of the renal sexual segment of male lizards, *Anolis carolinensis*. Herpetologica 28: 53-57.

Darwin C (1871). The Descent of Man, and Selection in Relation to Sex. Murray: London.

DeCourcy KR, Jenssen TA (1994). Structure and use of male territorial headbob signals by the lizard *Anolis carolinensis*. Anim Behav 47: 251-262.

Dederen PJ, Gribnau AA, Curfs MH (1994). Retrograde neuronal tracing with cholera toxin B subunit: comparison of three different visualization methods. Histochem J 26: 856-862.

DeVoogd TJ, Pyskaty DJ, Nottebohm F (1991). Lateral asymmetries and testosterone-induced changes in the gross morphology of the hypoglossal nucleus in adult canaries. J Comp Neurol 307: 65-76.

Dorlochter M, Astrow SH, Herrera AA (1996). Effects of testosterone on a sexually dimorphic frog muscle: repeated *in vivo* observations and androgen receptor distribution. J Neurobiol 25: 897-916.

Dufaure JP, Hubert J (1961). Table de developpement du lezard vivipare: *Lacerta (zootoca) vivipara jacquin*. Arch Ant Micros Morphol Exp 50: 309-327.

Dunlap KD, Zakon HH (1998). Behavioral actions of androgens and androgen receptor expression in the electrocommunication system of an electric fish, *Eigenmannia virescens*. Horm Behav 34: 30-38.

Dunlap KD, McAnelly ML, Zakon HH (1997). Estrogen modifies an electrocommunication signal by altering the electrocyte sodium current in an electric fish, *Sternopygus*. J Neurosci 17: 2869-2875.

Dunlap KD, Thomas P, Zakon HH (1998). Diversity of sexual dimorphism in electrocommunication signals and its androgen regulation in a genus of electric fish, *Apteronotus*. J Comp Physiol [A] 183: 77-86.

Elbrecht A, Smith RB (1992). Aromotase enzyme activity and sex determination in chickens. Science 255: 467-70.

Fargo KN, Foster AM, Harty MW, Sengelaub DR (2003). Estrogen alters excitability but not morphology of a sexually dimorphic neuromuscular system in adult rats. J Neurobiol 56: 66-77.

Fischer LM, Catz D, Kelley DB (1995). Androgen-directed development of the Xenopus laevis larynx: control of androgen receptor expression and tissue differentiation. Dev Biol 170: 115-126.

Fishman RB, Chism L, Firestone GL, Breedlove SM (1990). Evidence for androgen receptors in sexually dimorphic perineal muscles of neonatal male rats: Absence of androgen acuumulation by the perineal motoneurons. J Neurobiol 21: 694-704.

Foa A, Magnone C, Bertolucci C (2002). Circadian organization in ruin lizards: phase response curve for melatonin changes with season. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 188:141-145.

Font E (1991). Localization of brainstem motoneurons involved in dewlap extension in the lizard, *Anolis equestris* (Iguanidae). Behav Brain Res 45: 171-176.

Font E, Rome LC (1990). Functional morphology of dewlap extension in the lizard *Anolis equestris* (Iguanidae). J Morph 206: 245-258.

Forbes TR (1964). Intersexuality in reptiles. In: C.N. Armstrong and A.J. Marhsall, editors. Intersexuality in Vertebrates, Including Man. Academic Press: New York, p. 273-283.

Forger NG, Breedlove SM (1987). Seasonal variation in mammalian striated muscle mass and motoneuron morphology. J Neurobiol 18: 155-165.

Forger NG, Fishman RB, Breedlove SM (1992). Differential effects of testosterone metabolites upon the size of sexually dimorphic motoneurons in adulthood. Horm Behav 26: 204-213.

Forlano PM, Sisneros JA, Knapp R, Bass AH (2003). Reproductive periodicity of gonadal steroid hormone levels in the vocal plainfin midshipman, *Porichthys notatus*. Horm Behav 44: 49.

Fraley GS, Ulibarri C (2001). Sexual dimorphism in the number and size of SNB motoneurons: delayed development during normal ontogeny. Dev Brain Res 126: 57-64.

Freeman LM, Padgett BA, Prins GS, Breedlove SM (1995). Distribution of androgen receptor immunoreactivity in the spinal cord of wild-type, androgen-insensitive and gonadectomized male rats. J Neurobiol 27: 51-59.

Ganesh S, Raman R (1995). Sex reversal by testosterone and not by estradiol or temperature in *Calotes versicolor*, the lizard lacking sex chromosomes. J Exp Zool 271: 139-144.

Girgenrath M, Marsh RL (2003). Season and testosterone affect contractile properties of fast calling muscles in the gray tree frog *Hyla chrysoscelis*. Am J Physiol 284: R1513-1520.

Glicksman MA, Chiu AY, Dionne CA, Harty M, Kaneko M, Murakata C, Oppenheim RW, Prevette D, Sengelaub DR, Vaught JL, Neff NT (1998). CEP-1347/KT7515 prevents motor neuronal programmed cell death and injury-induced dedifferentiation in vivo. J Neurobiol 35:361-370.

Godsave SF, Lohmann R, Vloet RPM, Gahr M (2002). Androgen receptors in the embryonic zebra finch hindbrain suggest a function for maternal androgens in perihatching survival. J Comp Neurol 453: 57-70.

Goldstein LA, Sengelaub DR (1990). Hormonal control of neuron number in sexually dimorphic spinal nuclei of the rat: IV. Masculinization of the spinal nucleus of the bulbocavernosus with testosterone metabolites. J Neurobiol 21: 719-730.

Goldstein LA, Sengelaub DR (1994). Differential effects of dihydrotestosterone and estrogen on the development of motoneuron morphology in a sexually dimorphic rat spinal nucleus. J Neurobiol 25: 878-892.

Gordon T, Thomas CK, Munson JB, Stein RB (2004). The resilience of the size principle in the organization of motor unit properties in normal and reinnervated adult skeletal muscles. Can J Physiol Pharmacol 82: 645-661.

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

Greenberg N, Scott M, Crews D (1984). Role of the amygdala in the reproductive and aggressive behavior of the lizard, *Anolis carolinensis*. Physiol Behav 32: 147-151.

Gunderson HJG (1986). Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompsen. J Microsc 143: 3-45.

Gurney ME (1981). Hormonal control of cell form and number in the zebra finch song system. J Neurosci 1: 658-673.

Hannigan P, Kelley DB (1986). Androgen-induced alterations in vocalizations of female Xenopus laevis: modifiability and constraints. J Comp Physiol [A] 158: 517-527.

Harding CF, Sheridan K, Walters MJ (1983). Hormonal specificity and activation of sexual behavior in male zebra finches. Horm Behav 17: 111-133.

Hart BL (1967). Testosterone regulation of sexual reflexes in spinal male rats. Science 155: 1282-1284.

Hart BL (1983). Role of testosterone secretion and penile reflexes in sexual behavior and sperm competition in male rats: A theoretical contribution. Physiol Behav 31: 823-827.

Hegstrom CD, Breedlove SM (1998). Short day lengths delay development of the SNB neuromuscular system in the Siberian hamster, *Phodopus sungorus*. J Neurobiol 35: 355-360.

Hegstrom CD, Breedlove SM (1999). Seasonal plasticity of neuromuscular junctions in adult male Siberian hamsters (*Phodopus sungorus*). Brain Res 819: 83-88.

Hegstrom CD, Jordan CL, Breedlove SM (2002). Photoperiod and androgens act independently to induce spinal nucleus of the bulbocavernosus neuromuscular

plasticity in the Siberian hamster, *Phodopus sungorus*. J Neuroendo 14: 368-374.

Hews DK, Moore MC (1995). Influence of androgens on differentiation of secondary sex characters in tree lizards, *Urosaurus ornatus*. Gen Comp Endo 97: 86-102.

Hews DK, Moore MC (1996). A critical period for the organization of alternative male phenotypes of tree lizards by exogenous testosterone? Physiol Behav 60: 425-429.

Hews DK, Knapp R, Moore MC (1994). Early exposure to androgens affects adult expression of alternative male types in tree lizards. Horm Behav 28: 96-115.

Holmes MM, Wade J (2004a). Characterization of projections from a sexually dimorphic motor nucleus in the spinal cord of adult green anoles. J Comp Neurol 471: 180-187.

Holmes MM, Wade J (2004b). Seasonal plasticity in the copulatory neuromuscular system of green anole lizards: a role for testosterone in muscle but not motoneuron morphology. J Neurobiol 60: 1-11.

Holmes MM, Putz O, Crews D, Wade J (2005). Normally occurring intersexuality and testosterone induced plasticity in the copulatory system of adult leopard geckos. Horm Behav 47: 439-445.

Holmes MM, Wade J (2005). Sexual differentiation of the copulatory neuromuscular system in green anoles (*Anolis carolinensis*): normal ontogeny and manipulation of steroid hormones. J Comp Neurol: in press.

Holmes MM, Wade J. Testosterone regulates androgen receptor immunoreactivity in the copulatory, but not courtship, neuromuscular system in adult male green anoles. J Neuroendo: submitted.

Hull EM, Meisel RL, Sachs BD (2002). Male sexual behavior. In: Hormones, Brain and Behavior, Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, editors. New York: Academic Press, p. 3-137.

Hyde LL, Underwood H (1995). Daily melatonin infusions entrain the locomotor activity of pinealectomized lizards. Physiol Behav 58: 943-951.

Hyde LL, Underwood H (2000). Effects of melatonin administration on the circadian activity rhythm of the lizard *Anolis carolinensis*. Physiol Behav 71: 183-192.

Jenssen TA, Nunez SC (1998). Spatial and breeding relationships of the lizard, Anolis carolinensis: evidence of intrasexual selection. Behaviour 135: 981-1003.

Jenssen TA, Greenberg N, Hovde KA (1995). Behavioral profile of free-ranging male lizards, *Anolis carolinensis*, across breeding and post-breeding seasons. Herpetol Monogr 9: 41-62.

Jenssen TA, Congdon JD, Fischer RU, Estes R, Kling D, Edmands S, Berna H (1996). Behavioural, thermal, and metabolic characteristics of a wintering lizard (*Anolis carolinensis*) from South Carolina. Func Ecol 10: 201-209.

Jenssen TA, Orrell KA, Lovern MB (2000). Sexual dimorphisms in aggressive signal structure and use by a polygynous lizard, *Anolis carolinensis*. Copeia 1: 140-149.

Jenssen TA, Lovern MB, Congdon JD (2001). Field-testing the protandry-based mating system for the lizard, *Anolis carolinensis*: does the model organism have the right model? Behav Ecol Sociobiol 50: 162-172.

Jordan CL (1997). Androgen receptor (AR) immunoreactivity in rat pudendal motoneurons: implications for accessory proteins. Horm Behav 32: 1-10.

Jordan CL, Breedlove SM, Arnold AP (1991). Ontogeny of steroid accumulation in spinal lumbar motoneurons of the rat: Implications for androgen's site of action during synapse elimination. J Comp Neurol 313: 441-448.

Jordan CL, Padgett G, Hershey J, Prins G, Arnold A (1997). Ontogeny of androgen receptor immunoreactivity in lumbar motoneurons and in the sexually dimorphic levator ani muscle of male rats. J Comp Neurol 379: 88-98.

Kay JN, Hannigan P, Kelley DB (1999). Trophic effects of androgen: development and hormonal regulation of neuron number in a sexually dimorphic vocal motor nucleus. J Neurobiol 40: 375-385.

Keizer K, Kuypers HG, Huisman AM, Dann O (1983). Diamidino yellow dihydrochloride (DY.2HCl); a new fluorescent retrograde neuronal tracer, which migrates only very slowly out of the cell. Exp Brain Res 51: 179-191.

Kelley DB (1986). Neuroeffectors for vocalization in *Xenopus laevis*: hormonal regulation of sexual dimorphism. J Neurobiol 17: 231-248.

Kelley DB, Pfaff DW (1976). Hormone effects on male sex behavior in adult South African clawed frogs, *Xenopus laevis*. Horm Behav 7: 159-182.

Kelley DB, Dennison J (1990). The vocal motor neurons of *Xenopus laevis*: development of sex differences in axon number. J Neurobiol 21: 869-882.

Kelley DB, Tobias ML (1999). The vocal repertoire of *Xenopus laevis*. In: The Design of Animal Communication, Hauser M, Konishi M, editors. MIT Press: Cambridge, p. 9-35.

Kelley DB, Fenstemaker S, Hannigan P, Shih S (1988). Sex differences in the motor nucleus of cranial nerve IX-X in Xenopus laevis: a quantitative Golgi study. J Neurobiol 19: 413-429.

Kelly KK, Goldman BD, Zucker I (1994). Gonadal growth and hormone concentrations in photoregressed Siberian hamsters: pinealectomy versus photostimulation. Biol Reprod 51: 1046-1050.

Knapp R, Marchaterre MA, Bass AH (1999). Early development of the motor and premotor circuitry of a sexually dimorphic vocal pathway in a teleost fish. J Neurobiol 38: 475-490.

Kuypers HG, Bentivoglio M, Catsman-Berrevoets CE, Bharos AT (1980). Double retrograde neuronal labeling through divergent axon collaterals, using two fluorescent tracers with the same excitation wavelength which label different features of the cell. Exp Brain Res 40: 383-392.

Lee JS, Bass AH (2005). Differential effects of 11-ketotestosterone on dimorphic traits in a teleost with alternative male reproductive morphs. Horm Behav 47: 523-531.

Leslie M, Forger NG, Breedlove SM (1991). Does androgen affect axonal transport of cholera toxin HRP in spinal motoneurons? Neurosci Lett 126: 199-202.

Ligon JD (1968). Sexual differences in foraging behavior in two species of *Dendrocopus* woodpeckers. Auk 85: 203-215.

Lovern MB, Jenssen TA (2001). The effects of context, sex, and body size on staged social interactions in juvenile male and female green anoles (*Anolis carolinensis*). Behaviour 138: 1117-1135.

Lovern MB, Wade J (2001). Maternal plasma and egg yolk testosterone concentrations during embryonic development in green anoles (*Anolis carolinensis*). Gen Comp Endo 124: 226-235.

Lovern MB, McNabb FMA, Jenssen TA (2001). Developmental effects of testosterone on behavior in male and female green anoles (*Anolis carolinensis*). Horm Behav 39: 131-143.

Lovern MB, Holmes MM, Wade J (2004a). The green anole (*Anolis carolinensis*): a reptilian model for laboratory studies of reproductive morphology and behavior. ILAR J 45: 54-65.

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

Lu S, Simon NG, Wang Y, Hu S (1999). Neural androgen receptor regulation: effects of androgen and antiandrogen. J Neurobiol 41: 505-512.

Mason P, Adkins EK (1976). Hormones and social behavior in the lizard, *Anolis carolinensis*. Horm Behav 7: 75-86.
McKenna KE, Nadelhaft I (1986). The organization of the pudendal nerve in the male and female rat. J Comp Neurol 248: 532-549.

Meek LR, Romeo RD, Novak CM, Sisk CL (1997). Actions of testosterone in prepubertal and postpubertal male hamsters: dissociation of effects on reproductive behavior and brain androgen receptor immunoreactivity. Horm Behav 31: 75-88.

Mills A, Zakon HH, Marchaterre MA, Bass AH (1992). Electric organ morphology of *Sternopygus macrurus*, a wave-type, weakly electric fish with a sexually dimorphic EOD. J Neurobiol 23: 920-932.

Monks DA, O'Bryant EL, Jordan CL (2004). Androgen receptor immunoreactivity in skeletal muscle: enrichment at the neuromuscular junction. J Comp Neurol 473: 59-72.

Morgantaler A, Crews D (1978). Role of the anterior hypothalamic-preoptic area in the regulation of reproductive behavior in the lizard, *Anolis carolinensis*: implantation studies. Horm Behav 11: 61-73.

Nakano H, Masuda K, Sasaki SY, Katsuta S (1997). Oxidative enzyme activity and soma size in motoneurons innervating the rat slow-twitch and fast-twitch muscles after chronic activity. Brain Res Bull 43: 149-154.

Nakano H, Katsuta S (2000). Non-weight bearing condition arrests the morphological and metabolic changes of rat soleus motoneurons during postnatal growth. Neurosci Lett 290: 145-148.

Nelson RJ (2000). An Introduction to Behavioral Endocrinology. Sinauer Associates: Massachusetts.

Nordeen EJ, Nordeen KW, Sengelaub D, Arnold AP (1985). Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus. Science 229: 671-673.

Nottebohm F, Arnold AP (1976). Sexual dimorphism in vocal control areas of the songbird brain. Science 194: 211-213.

Nottebohm F, Stokes TM, Leonard CM (1976). Central control of song in the canary, *Serinus canaries*. J Comp Neurol 165: 457-486.

Nunez SC, Jenssen TA, Ersland K (1997). Female activity profile of a polygynous lizard (*Anolis carolinensis*): evidence of intersexual asymmetry. Behaviour 134: 205-223.

O'Bryant EL, Wade J (1999). Sexual dimorphisms in a neuromuscular system regulating courtship in the green anole lizard: Effects of season and androgen treatment. J Neurobiol 40: 202-213.

O'Bryant EL, Wade J (2000). Arborization of dewlap motoneurons in the green anole lizard (*Anolis carolinensis*) is not sexually dimorphic. Neurosci Letters 281: 115-118.

O'Bryant EL, Wade J (2001). Development of a sexually dimorphic neuromuscular system involved in green anole courtship behavior. Brain Behav Evol 58: 362-369.

O'Bryant EL, Wade J (2002a). Seasonal and sexual dimorphisms in the green anole forebrain. Horm Behav 41: 384-395.

O'Bryant EL, Wade J (2002b). Sexual dimorphism in neuromuscular junction size on a muscle used in courtship by green anole lizards. J Neurobiol 50: 24-30.

Ohmori Y, Watanabe T (1989). Location of motoneurons innervating the musculus sphincter cloacae in the domestic fowl. Neurosci Lett 101: 1-5.

Park KH, Meitzen J, Moore IT, Brenowitz EA, Perkel DJ. Seasonal-like plasticity of spontaneous firing rate in a songbird pre-motor nucleus. J Neurobiol: in press.

Pasmanik M, Callard GV (1988). A high abundance of androgen receptor in goldfish brain: characteristics and seasonal changes. Endocrinology 123: 1162-1171.

Perez J, Kelley D (1996). Trophic effects of androgen: receptor expression and the survival of laryngeal motor neurons after axotomy. J Neurosci 16: 6625-6633.

Phoenix CH, Goy RW, Gerall AA, Young WC (1959). Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology 65: 369-382.

Pough FH, Andrews RM, Cadle JE, Crump ML, Savitzky AH, Wells KD (2001). Herpetology, 2nd edition. Prentice Hall: New Jersey.

Rand MN, Breedlove SM (1987). Ontogeny of functional innervation of bulbocavernosus muscles in male and female rats. Brain Res 430: 150-152.

Rand MN, Breedlove SM (1992). Androgen locally regulates rat bulbocavernosus and levator ani size. J Neurobiol 23: 17-30.

Rand MN, Breedlove SM (1995). Androgen alters the dendritic arbors of SNB motoneurons by acting upon their target muscles. J Neurosci 15: 4408-4416.

Raouf S, Van Roo B, Seglaub F (2000). Adult plasticity in hormone-sensitive motoneuron morphology: methodological/behavioral confounds. Horm Behav 38: 210-221.

Raynaud A, Pieau C (1985). Embryonic development of the genital system. In: Biology of the Reptilia, Gans C editor. John Wiley & Sons: USA.

Rhen T, Crews D (2001). Distribution of androgen and estrogen receptor mRNA in the brain and reproductive tissues of the leopard gecko, *Eublepharis macularius*. J Comp Neurol 437: 385-397.

Rhen T, Ross J, Crews D (1999). Effects of testosterone on sexual behavior and morphology in adult female leopard geckos, *Eublepharis macularius*. Horm Behav 36: 119-128.

Rhen T, Sakata JT, Zeller M, Crews D (2000). Sex steroid levels across the reproductive cycle of female leopard geckos, *Eublepharis macularius*, from different incubation temperatures. Gen Comp Endo 118: 322-331.

Rhen T, Sakata JT, Woolley S, Porter R, Crews D (2003). Changes in androgen receptor mRNA expression in the forebrain and oviduct during the reproductive cycle of female leopard geckos, *Eublepharis macularius*. Gen Comp Endo 132: 133-141.

Robertson JC, Watson JT, Kelley DB (1994). Androgen directs sexual differentiation of laryngeal innervation in developing *Xenopus laevis*. J Neurobiol 25: 1625-1636.

Romeo RD, Richardson HN, Sisk CL (2002). Puberty and the maturation of the male brain and sexual behavior: recasting a behavioral potential. Neurosci Biobehav Rev 26: 381-391.

Rosen GJ, Wade J (2000). The role of 5-alpha-reductase activity in sexual behaviors of the green anole lizard. Physiol Behav 69: 487-498.

Rosen GJ, O'Bryant EL, Matthews J, Zacharewski T, Wade J (2002). Distribution of androgen receptor mRNA expression and immunoreactivity in the brain of the green anole lizard. J Neuroendo 14: 19-28.

Ruby DE (1984). Male breeding success and differential access to females in *Anolis carolinensis*. Herpetologica 40: 272-280.

Ruiz CC, Wade J (2002). Sexual dimorphisms in a copulatory neuromuscular system in the green anole lizard. J Comp Neurol 443: 289-297.

Sassoon D, Kelley DB (1986). The sexually dimorphic larynx of *Xenopus laevis*: development and androgen regulation. Am J Anat 177: 457-472.

Sassoon D, Segil N, Kelley D (1986). Androgen-induced myogenesis and chondrogenesis in the larynx of *Xenopus laevis*. Dev Biol 113: 135-140.

Sassoon DA, Gray GE, Kelley DB (1987). Androgen regulation of muscle fiber type in the sexually dimorphic larynx of *Xenopus laevis*. J Neurosci 7: 3198-3206.

Seiwert CM, Adkins-Regan E (1998). The foam production system of the male Japanese quail: Characterization of structure and function. Brain Behav Evol 52: 61-80.

Selander RK (1966). Sexual dimorphism and differential niche utilization in birds. Condor 68: 113-151.

Schroder HD (1980). Organization of the motoneurons innervating the pelvic muscles of the male rat. J Comp Neurol 192: 567-587.

Siegford JM, Ulibarri C (2004). Increase in motoneurons in the spinal nucleus of the bulbocavernosus of prepubertally castrated male Mongolian gerbils following delayed treatment with testosterone. J Comp Neurol 472: 485-495.

Simpson HB, Tobias ML, Kelley DB (1986). Origin and identification of fibers in the cranial nerve IX-X complex of *Xenopus laevis*: Lucifer yellow backfills in vitro. J Comp Neurol 244: 430-444.

Sinha-Hikim I, Taylor WE, Gonzalez-Cadavid NF, Zheng W, Bhasin S (2004). Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. J Clin Endocrinol Metab 89: 5245-5255.

Sisneros JA, Bass AH (2003). Seasonal plasticity of peripheral auditory frequency sensitivity. J Neurosci 23: 1049-1058.

Soma KK, Hartman VN, Wingfield JC, Brenowitz EA (1999). Seasonal changes in androgen receptor immunoreactivity in the song nucleus HVc of a wild bird. J Comp Neurol 409: 224-236.

Souccar C, Lapa AJ, Ribeiro do Valle J (1982). Influence of castration on the electrical excitability and contraction properties of the rat levator ani muscle. Exp Neurol 75: 576-588.

Sperry TS, Thomas P (1999). Characterization of two nuclear androgen receptors in Atlantic croaker: comparison of their biochemical properties and binding specificities. Endocrinology 104: 1602-1611.

Springer ML, Wade J (1997). The effects of testicular tissue and prehatching inhibition of estrogen synthesis on the development of courtship and copulatory behavior in zebra finches. Horm Behav 32: 46-59.

Tobias ML, Kelley DB (1995). Sexual differentiation and hormonal regulation of the laryngeal synapse in *Xenopus laevis*. J Neurobiol 28: 515-526.

Tobias ML, Marin ML, Kelley DB (1991). Temporal constraints on androgen directed laryngeal masculinization in *Xenopus laevis*. Dev Biol 147: 260-270.

Tobias ML, Tomasson J, Kelley DB (1998). Attaining and maintaining strong vocal synapses in female *Xenopus laevis*. J Neurobiol 37: 441-448.

Tokarz RR (1988). Copulatory behavior of the lizard *Anolis sagrei*: alternation of hemipenis use. Anim Behav 36: 1518-1524.

Tokarz RR, Slowinski JB (1990). Alternation of hemipenis use as a behavioural means of increasing sperm transfer in the lizard *Anolis sagrei*. Anim Behav 40: 374-379.

Tokarz RR, Kirkpatrick SJ (1991). Copulation frequency and pattern of hemipenis use in males of the lizard *Anolis sagrei* in a semi-natural enclosure. Anim Behav 41: 1039-1044.

Tokarz RR, McMann S, Seitz L, John-Alder H (1998). Plasma corticosterone and testosterone levels during the annual reproductive cycle of male brown anoles (*Anolis sagrei*). Physiol Zool 71: 139-146.

Tokarz RR, McMann S, Smith LC, John-Alder H (2002). Effects of testosterone treatment and season on the frequency of dewlap extensions during male-male interactions in the lizard *Anolis sagrei*. Horm Behav 41: 70-79.

Tousignant A, Crews D (1994). Effect of exogenous estradiol applied at different embryonic stages on sex determination, growth, and mortality in the leopard gecko (*Eublepharis macularius*). J Exp Zool 268: 17-21.

Veney SL, Wade J (2004). Steroid receptors in the adult zebra finch syrinx: a sex difference in androgen receptor mRNA, minimal expression of estrogen receptor α and aromatase. Gen Comp Endo 136: 192-199.

Veney SL, Wade J (2005). Post-hatching syrinx development in the zebra finch: an analysis of androgen receptor, aromatase, estrogen receptor alpha and estrogen receptor beta mRNAs. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 191: 97-104.

Viets BE, Ewert MA, Talent LG, Nelson CE (1994). Sex-determining mechanisms in squamate reptiles. J Exp Zool 270: 45-56.

Wade J (1998). Sexual dimorphisms in the brainstem of the green anole lizard. Brain Behav Evol 52: 46-54.

Wade J (1999). Sexual dimorphisms in avian and reptilian courtship: Two systems that do not play by mammalian rules. Brain Behav Evol 54: 15-27.

Wade J (2001). Zebra finch sexual differentiation: the aromatization hypothesis revisited. Microsc Res Tech 54: 354-363.

Wade J (2005). Current research on the behavioral neuroendocrinology of reptiles. Horm Behav: in press.

Wade J, Arnold AP (1996). Functional testicular tissue does not masculinize development of the zebra finch song system. Proc Natl Acad Sci 93:5264-5268.

Wade J, Buhlman L (2000) Lateralization and effects of adult androgen in a sexually dimorphic neuromuscular system controlling song in zebra finches. J Comp Neurol 426:154-164.

Wade J, Arnold AP (2004). Sexual differentiation of the zebra finch song system. Ann NY Acad Sci 1016: 1-20.

Wade J, Gong A, Arnold AP (1997). Effects of embryonic estrogen on differentiation of the gonads and secondary sexual characteristics of male zebra finches. J Exp Zool 278: 405-411.

Wade J, Swender DA, McElhinny TL (1999). Sexual differentiation of the zebra finch song system parallels genetic, not gonadal, sex. Horm Behav 36: 141-152.

Wade J, Buhlman L, Swender D (2002). Post-hatching hormonal modulation of a sexually dimorphic neuromuscular system controlling song in zebra finches. Brain Res 929: 191-201.

Watson JT, Kelley DB (1992). Testicular masculinization of vocal behavior in juvenile female *Xenopus laevis* reveals sensitive periods for song duration, rate, and frequency spectra. J Comp Physiol [A] 171: 343-350.

Watson JT, Robertson J, Sachdev U, Kelley DB (1993). Laryngeal muscle and motor neuron plasticity in *Xenopus laevis*: testicular masculinization of a developing neuromuscular system. J Neurobiol 24: 1615-1625.

Wetzel DM, Kelley DB (1983). Androgen and gonadotropin effects on male mate calls in South African clawed frogs, *Xenopus laevis*. Horm Behav 17: 388-404.

Wetzel DM, Haerter UL, Kelley DB (1985) A proposed neural pathway for vocalization in South African clawed frogs, *Xenopus laevis*. J Comp Physiol [A] 157: 749-761.

Wibbels T, Crews D (1992). Specificity of steroid hormone-induced sex determination in a turtle. J Endocrinol 133: 121-129.

Wibbels T, Crews D (1995). Steroid-induced sex determination at incubation temperatures producing mixed sex ratios in a turtle with TSD. Gen Comp Endo 100: 53-60.

Winkler SM, Wade J (1998). Aromatase activity and regulation of sexual behaviors in the green anole lizard. Physiol Behav 64: 723-731.

Zakon HH, Dunlap KD (1999). Sex steroids and communication signals in electric fish: a tale of two species. Brain Behav Evol 54: 61-69.