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Sexual Dimorphisms in a Copulatory Neurmuscular System in the Green Anole Lizard

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SEXUAL DIMORPHISMS IN A COPULATORY NEUROMUSCULAR SYSTEM IN THE GREEN ANOLE LIZARD

By

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ABSTRACT

SEXUAL DIMORPHISMS IN A COPULATORY NEUROMUSCULAR SYSTEM IN THE GREEN ANOLE LIZARD

By

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Sexual dimorphisms in neuromuscular systems have been investigated in several vertebrate groups, but data on reptiles is limited. The present studies were designed to establish the copulatory neuromuscular system of the green anole lizard as an appropriate model. Like mammals, male reptiles have copulatory organs. However, each individual has two "hemipenes" that are controlled by bilateral sets of muscles. First, the anatomy of these structures was examined in males and the same anatomical region in females. Spinal motoneurons innervating one of these muscles, the transversus penis (TPN), were localized using the retrograde tracer biocytin and were detected in the last trunk and first sacral segments (T17-S1). Motoneuron number and size were assessed in Nissl-stained sections containing T17-S1 in adult males and females. Male-biased sexual dimorphisms were detected in both measures, but the motoneurons innervating the caudifemoralis (CF) tail muscle are also located in T17-S1. Therefore, the CF motoneurons were localized and eliminated from the analysis in order to gain a more accurate representation of the TPN motoneuron pool. An equivalent number of these cells were labeled in both sexes, and the results from the previous study were replicated. Thus, similar to other vertebrate models, parallels between morphology and function exist in the green anole copulatory system. Future investigations will broaden the comparative perspective on mechanisms regulating sexual dimorphisms relating to reproductive behaviors in vertebrates.

ACKNOWLEDGMENTS

We thank Stephen Schneider for help with the lizard vertebrate anatomy and Erin O'Bryant and Michelle Smith for technical assistance. Figure 1 was drawn by Marlene Cameron. This work was supported by National Science Foundation grant IBN-9733074 to Juli Wade and a Michigan State University Minority Competitive Doctoral Fellowship to Claudia C. Ruiz.

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ABBREVIATIONS

CF.....caudifemoralis

IC.....ischiocaudalis

ILC.....iliocaudalis

HCL.....hydrochloric acid

PBF.....10% phosphate buffered formalin

PBS.....0.1 M phosphate buffered saline

RPM.....retractor penis magnus

SNB.....spinal nucleus of the bulbocavernosus

T17-S1.....trunk segment 17 through sacral segment 1

TPN.....transversus penis

XIIts.....tracheosyringeal portion of the hypoglossal nucleus

INTRODUCTION

The relationship between structure and function has been investigated in diverse vertebrate models, including neuromuscular systems that regulate courtship and copulation. For example, adult male zebra finches court females by singing, and females do not sing. In parallel, the tracheosyringeal portion of the hypoglossal nucleus (XIIts) is larger in male than female songbirds, and fibers in its target muscles in the vocal organ (syrinx) are also larger in males (Nottebohm and Arnold, 1976; DeVoogd and Nottebohm, 1981; Wade and Buhlman, 2000). Male African clawed frogs (*Xenopus laevis*) also use vocalizations during courtship (Russell, 1954). They have more motoneurons than females in the cranial nerve nucleus IX-X, which innervates the larynx muscles (Kelley, 1986). Neuron soma size, laryngeal muscle mass and fiber size are also increased in males compared to females (Sassoon and Kelley, 1986; Kelley et al., 1988). Similarly, some male midshipman fish (*Porichthys notatus*) vocalize to females during courtship. A group of motoneurons in the caudal brainstem innervate the sonic muscle in the walls of the swim bladder (reviewed in Bass, 1990) and the soma size and number of these cells are greater in Type I males, who vocalize, than in females and Type II males, who do not (Bass and Marchaterre, 1989a; Bass and Baker, 1990). Type I males also have heavier sonic muscles with larger fibers, compared to females and Type II males (Bass and Marchaterre, 1989b). Like these vertebrate courtship models, a relatively simple neuromuscular system is involved in mammalian copulation. The spinal nucleus of the bulbocavernosus (SNB) contains motoneurons innervating the bulbocavernosus (BC) and levator ani (LA) muscles, which modulate penis function (Hart and Melese-d'Hospital, 1983). The SNB motoneurons are larger and more numerous in males than in

females, and the muscles are present in males, but have regressed in females (Breedlove and Arnold, 1980).

The green anole lizard (*Anolis carolinensis*) is also emerging as a useful model for the investigation of sex differences in structure and function. Males extend a red throat fan (dewlap) during courtship (Greenberg and Noble, 1944; Crews, 1979). The paired ceratohyoid muscles, located in the throat, control dewlap extension and are innervated by motoneurons in the caudal brainstem (Font, 1991; Wade, 1998). A variety of male biased dimorphisms have been identified in this system, including motoneuron, nerve and muscle fiber size (Wade, 1998; O'Bryant and Wade, 1999). Importantly, this lizard species offers a second model for studying the relationship between structure and function. Because reptiles, like mammals, have copulatory organs, the neuromuscular control of copulation can be investigated in addition to that regulating courtship. This opportunity provides a unique advantage in elucidating the consistencies of mechanisms regulating sex differences of morphology and behavior, since previously courtship and copulatory systems had been explored in different vertebrate groups.

Male lizards have bilateral hemipenes, rather than a single penis, which are independently controlled (Arnold, 1984). Function of each hemipene is facilitated by two major muscles, the transversus penis (TPN) and the retractor penis magnus (RPM). The TPN is a thin, broad, superficial muscle that wraps around the ventral side of each hemipene as it lays in the tail just caudal to the cloaca (Figure 1C). During copulation, contraction of the TPN everts the hemipene through the vent of the cloaca (Arnold, 1984). The RPM runs from caudal vertebrae to insert on the posterior end of the hemipene and facilitates retraction into the cloaca following copulation (Arnold, 1984).

The RPM is a long, thin cylindrical muscle, lying underneath the ilio-ischiocaudalis complex, which consists of the ishiocaudalis (IC) and iliocaudalis (ILC). The IC and ILC are relatively large muscles that move the tail (Arnold, 1984).

The present study was conducted to begin to establish this neuromuscular system as a new reptilian model for the investigation of structure/function relationships. Specifically, the goals were to (1) describe the relevant anatomy in the green anole, including determining whether females possess hemipene muscles similar to males; (2) localize the motoneurons in the spinal cord innervating the hemipene muscles that facilitate copulation; and (3) determine whether these motoneurons are sexually dimorphic in size and number.

MATERIALS AND METHODS

Four separate studies were conducted. **Study 1:** The gross anatomy of the vertebral column, spinal cord and hemipene muscles in males and females were investigated because, to our knowledge, detailed anatomical descriptions were not available for the green anole lizard. Histological preparations of the rostral tail were also prepared in both sexes, which in males contain the hemipenes and associated muscles. **Study 2:** Biocytin was used to determine the location of the motoneurons innervating the TPN in males (the RPM was separately injected in a number of individuals, but despite various modifications of the technique, motoneuron somata were not successfully labeled in any region of the spinal cord). **Study 3:** Nissl-stained tissue was used to determine whether the anatomy of the spinal cord in the region containing TPN motoneurons was sexually dimorphic during the breeding season. **Study 4:** A preliminary experiment

determined that motoneurons projecting to the caudifemoralis, the principle leg muscle responsible for retracting the thigh (CF; Snyder, 1954) are located in the same region of the spinal cord as the TPN motoneurons. Biocytin was used to identify those cells in both sexes so that they could be eliminated from a replicate investigation of sexual dimorphisms in the spinal cord with the goal of increasing the specificity of the analysis to the motoneurons involved in copulation.

Procedures were approved by the Michigan State University All University Committee on Animal Use and Care and conform to NIH guidelines.

Animals and Housing

Recently captured adult male and female green anoles were purchased from a commercial supplier (Fluker Farms, Port Allen, LA). Initially, one adult male and three to seven adult females were housed in 110-liter glass aquaria with peat moss substrate and sticks and rocks for climbing and basking. Animals were housed on a 14:10 h light cycle using fluorescent and full spectrum lights. Ambient temperature ranged from 18°C during the night to 28-38°C during the day depending on the proximity to a heat lamp located over each cage. Aquaria were sprayed with water daily to maintain 70% relative humidity, and water was provided *ad libitum*. The diet consisted of mealworms or vitamin-dusted crickets fed three times per week. For Study 1, tissue was collected from breeding males and females in group housing conditions. In Study 2, individual males were placed in a 38-liter aquarium immediately following biocytin injections. For Study 3, male and female pairs were transferred from group housing conditions to 38-liter aquaria six to seven days before sacrifice. In Study 4, pairs were transferred from group

housing conditions to 38-liter aquaria immediately following biocytin injections, six to seven days before sacrifice.

Tissue Collection, Processing and Analysis

Study 1- Examination of General Anatomy

In order to precisely determine the segments of the anole spinal cord, we compared 6 green anole skeletal specimens (borrowed from the Michigan State University Museum Collection) to more general reptilian atlases and anatomical descriptions (Romer, 1956; Hoffstetter and Gasc, 1969). Six males from our colony were given an overdose of Sodium Brevital (Eli Lilly and Co.) and dissected to provide information on the gross anatomy of the soft tissues. Then, six pairs of adult male and female lizards where sacrificed in the same manner. Their breeding condition was confirmed by observation of medium to large testes with distinct tubules and hypertrophied vasa deferentia in males and at least one medium to large yolking follicle in females. The portion of tail between the cloaca and the caudal end of the RPM muscle (in males, and in a comparable location in females) was placed in Bouin's fixative for 14 days. The tissue was then soaked overnight in 70% ethanol, dehydrated in a series of alcohols, cleared in xylene and embedded in paraffin. The tissue was cut at 10 μm, mounted on slides and stained with the trichrome method (Forger et al., 1995). The sections were examined on an Olympus BX60 microscope and compared to a general description of lizard cloacal and hemipene anatomy (Arnold, 1984).

Study 2-Localization of Motoneurons

While deeply anesthetized with Isoflurane, the TPN muscles of 7 adult males were injected with 2 µl of 2% biocytin (Sigma) in 0.1 M phosphate buffered saline (PBS) 3 to 4 times throughout the muscle (6-8 µl total). Excess biocytin was picked up with a cotton swab, and the incision was sutured with silk. After 5-6 days, animals were given a lethal dose of Sodium Brevital. They were perfused intracardially with PBS followed by 4% paraformaldehyde/1% glutaraldehyde. The breeding condition of these males was confirmed as in Study 1. The vertebral column was removed from the body and postfixed in 4% paraformaldehyde/1% glutaraldehyde for one hour. The segments were marked with India ink on the animal's right side at the points where the dorsal roots enter the column, and the spinal cord was removed. Cords were embedded in sucrose/gelatin and sunk in 20% sucrose/4% paraformaldehyde overnight at 4°C. Frozen 30µm horizontal sections of 5 animals and 30µm cross-sections of 2 animals were cut into PBS.

Retrograde transport of biocytin into motoneurons was visualized as follows. The sections were rinsed in two changes of PBS at room temperature for 10 minutes each. They were then incubated in 0.1 M PBS-Triton X (0.3%) for 30 minutes followed by avidin-biotin complex (ABC; Vector Elite kit) in PBS for 3 hours. Tissue was washed in PBS for 10 minutes and then in 0.05 M Tris-HCl twice for 10 minutes each. Sections were then placed in 0.05% diaminobenzidine (Sigma) containing 0.025% H_2O_2 for 12-16 minutes, followed by two 10 minute washes in PBS. The tissue was mounted on gelatin covered slides from 9:1 dH_20:PBS and dried overnight. It was lightly counter-stained with thionin and coverslipped with Permount (Fisher).

Study 3-Quantification of Motoneurons

Nine pairs of breeding male and female lizards were given an overdose of Sodium Brevital and perfused intracardially with PBS followed by 10% phosphate buffered formalin (PBF). The breeding condition of animals was confirmed as in Study 1. Based on the results of Study 1, the portion of the vertebral column containing trunk segments 16-17, sacral segments 1-2 and the first caudal segment were removed from the body and postfixed in PBF for one hour. The spinal cord was marked and removed as in Study 2. Cords were embedded in sucrose/gelatin and sunk in a 20% sucrose/10% PBF solution overnight at 4°C. Frozen cross-sections were cut at 30μm and stained with thionin. The tissue from two females could not be used due to histological artifact. In all other animals, motoneuron number and soma size were assessed by an individual blind to their sex in the region of the spinal cord containing biocytin labeling from Study 1 (trunk segment 17 and sacral segment 1). These multipolar motoneurons are easily identifiable as they have large somata, stain densely, and are located approximately in a row oriented toward the central canal in the lateral portion of the ventral horn. The neurons just dorsomedial to them are smaller and more randomly clustered, so they can be easily distinguished. The total number of the motoneurons of interest was counted in every other section on each side of the spinal cord using the physical dissector technique (Gunderson, 1986). This procedure consists of counting only nuclei that come into focus and disappear within a single section, which insures that no neuron is counted twice. Soma size was measured in 20 randomly selected motoneurons each on the left and right sides of the spinal cord using NIH Image, and an average value for each side was analyzed for each animal. For a control, neuron soma size was assessed with the same

technique in trunk segment 16, where biocytin-labeled cells were never detected. The morphology of these neurons was also distinct from that of cells projecting to the hemipene muscles; they appeared smaller in size, did not stain as intensely, and were not oriented in a row. Motoneuron number and soma size were analyzed by ANOVA (Statview, SAS Institute). In addition to determining whether main effects of sex existed, potential differences between the left and right sides of the cord were assessed within individuals. Differences were considered significant if $p<0.05$.

Study 4-Elimination of Caudifemoralis Motoneurons from T17-S1

Ten pairs of adult male and female anoles were anesthetized with Isoflurane. The left CF muscles were injected with 1 µl of 2% biocytin in PBS each in the anterior, middle and posterior portion of the muscle (3 µl total). Excess biocytin was picked up with a cotton swab, and the incision was sutured. After 6-7 days, animals were given an overdose of Sodium Brevital and perfused intracardially as in Study 1. The breeding condition of all animals was confirmed as in Study 1. The portion of the vertebral column containing trunk segments 13 through 17 and sacral segments 1 and 2 were marked and removed from the body, and the tissue was processed as in Study 2 (horizontal sections). The tissue from three males could not be used due to histological artifact. In all other animals, motoneuron number and soma size were assessed in Nissl-stained cells (those not labeled with biocytin) as in Study 3. The motoneurons were as easy to identify in horizontal sections as in coronal sections due to their characteristic morphology and location in T17-S1. The change in plane of section between studies 3 and 4 was made because we did not want to risk losing or incorrectly sequencing the

large number of extremely small coronal sections that we would have to process to visualize the biocytin (even if every other section was used). Nissl-stained motoneuron number and soma size were analyzed as in Study 3, except that cells were counted in every section. Biocytin-labeled cells could not be accurately counted in T17-S1 using the technique employed for the Nissl-stained cells because the labeling was too intense and homogenous to see the nucleus or nucleolus. Also, the CF motoneuron somata frequently overlapped each other, making it difficult to distinguish individual cells. However, soma size was measured in 10 distinct biocytin-labeled cells in each individual, except in one male and one female where only 3 or 4 neurons, respectively, could be assessed. Fewer cells were analyzed in those 2 individuals because due to the degree of overlap, we could not be confident that the same cell would not be measured twice. The average soma size in biocytin-labeled cells was compared between the sexes by two-tailed t-test.

RESULTS

Study 1--Examination of General Anatomy

Lizard spinal segments are divided into four categories; *cervical*, *trunk* or *dorsal*, *sacral* and *caudal* (Romer, 1956; Hoffstetter and Gasc, 1969). Segments corresponding with the neck are considered *cervical*, those bearing ribs and connecting with the ilium are termed *sacral*, and segments of the tail that are posterior to the ilium are *caudal*. The anterior portion of the *trunk* segments bear ribs that fuse with the sternum while the posterior segments bear shorter ribs that support the weight of the trunk. *Anolis carolinensis* has 7 cervical, 17 trunk and 2 sacral segments (Figure 1A, 1B). With the specimens available, it was impossible to get an accurate count of the caudal vertebrae.

Histological sections of tail tissue in males revealed distinct hemipenes and TPN and RPM muscles (Figure 1C, 2A), but none of these structures were detected in females (Figure 2B).

Study 2-Localization of Motoneurons

Motoneurons projecting to the TPN muscle were consistently identified by intense brown biocytin labeling in the lateral portion of the ventral horn in the region containing the last trunk segment (T17) and the first sacral segment (S1) in all males (Figure 3A, 3B). Additional labeling, that was substantially lighter, was found in 4 animals at trunk segments 13-15, and in 2 of those animals also at trunk segments 11-12.

Study 3-Quantification of Motoneurons

Males had significantly more motoneurons in T17-S1 than females (F=12.55, p=0.003; Figure 4A, 5A, 5B). An equivalent number of cells was present on the two sides of the spinal cord (F=4.16, p=0.061), and the side and sex interaction was not significant (F=0.34, p=0.542). Similarly, males had significantly larger neuron somata in T17-S1 than females (F=4.95, p=0.043; Figure 4B), but a significant effect of side (F=0.02, p=0.888) and interaction (F=0.510, p=0.493) did not exist. Males and females did not differ in motoneuron soma size in the control region (T16) (F=0.81, p=0.386; Figure 4C). The effect of side (F=1.07, p=0.320) and interaction (F=0.46, p=0.512) in this region were also not statistically significant.

Study 4-Elimination of Caudifemoralis Motoneurons from T17-S1

In comparisons of Nissl-stained motoneurons, males had significantly more cells in T17-S1 than females (F=68.99, p<0.0001; Figure 6A, 7A, 7B). The right side of the spinal cord, contralateral to the biocytin injection, had more motoneurons stained only with thionin than the left (F=20.16, p=0.0004), but the interaction between side and sex was not significant (F=0.01, p=0.924; Figure 6A). Males also had significantly larger Nissl-stained neuron somata in T17-S1 than females (F=130.55, p <0.0001; Figure 6B). The cells were equivalent in size on the two sides of the cord (F=0.28, p=0.606), and the sex by side interaction was not statistically significant (F=0.51, p=0.488). Soma size in biocytin-labeled cells was equivalent in the two sexes (t=1.18, p=0.255).

DISCUSSION

Sex differences in the copulatory system of green anoles are striking. In adulthood, only males possess hemipenes and the associated muscles used to evert and retract the organs. Motoneurons in T17-S1, which includes those projecting to the TPN, were significantly larger and more numerous in males than in females, and that effect was confirmed when CF motoneurons were eliminated from the analysis. The larger soma size and greater neuron number in males compared to females in T17-S1 appears to be relatively specific to cells associated with copulation. For example, biocytin-labeled CF motoneurons were equivalent in size in the two sexes. Additionally, Nissl-stained motoneurons were equivalent in size in males and females in the spinal segment immediately rostral to T17-S1 (T16). The T16 cells were not counted because, unlike in

T17-S1, no distinct cell groups were obvious. However, it is clear that neuron number is not sexually dimorphic in all motor pools in the green anole. For example, in Study 4, an equivalent number of CF-projecting cells were labeled in the two sexes (they were not counted directly, but the difference in counts of cells stained only with thionin between the two sides was the same in males and females: $t= 0.097$, $p= 0.924$). In addition, motoneuron number in brainstem regions that project to the highly sexually dimorphic ceratohyoid muscle, which controls dewlap extension, is equivalent in males and females (O'Bryant and Wade, 1999).

However, even with the elimination of the CF motoneurons, a substantial number of cells in T17-S1 remained in females. Their target muscles are currently unknown. These muscles may be the same in the two sexes, and used for either similar or different purposes. In either case, one might predict that the motoneurons would be sexually monomorphic. This situation is similar to the Japanese quail, in which males produce foam during copulation from a gland under the tail (Seiwert and Adkins-Regan, 1998). Although this structure and the sphincter cloacae muscle, which facilitates use of the gland in males and oviposition in females, are sexually dimorphic, the motoneurons do not seem to differ between the sexes or between males of varying hormonal profiles (Seiwert and Adkins-Regan, 1998). Alternatively, the remaining T17-S1 motoneurons might innervate completely different muscles in males and females, but if these structures have equivalent levels of use, one would predict comparable motoneuron morphology and number in each sex. Finally, some of the motoneurons in males may project to the other hemipene muscles, such as the RPM, which is largely responsible for hemipene

retraction, and the retractor lateralis posterior, an extremely small muscle which may also aid in this function (Arnold, 1984).

Despite the present uncertainty in the targets of some of the T17-S1 neurons, the green anole is an excellent model in which to investigate relationships between structure and function and the mechanisms modulating both anatomy and behavior. To date, neuromuscular systems controlling copulation have been extensively investigated only in mammals and in detail only in rodents (Breedlove and Arnold, 1980; Forger and Breedlove, 1987; Freeman and Breedlove, 1995; Forger et al., 1996). The SNB contains larger and more numerous motoneurons in males compared to females, and the BC and LA muscles are greatly enhanced in males (Breedlove and Arnold, 1980). Perinatal testosterone preserves the BC and LA muscles, which in turn spare the SNB cells from cell death (Breedlove, 1984; Rand and Breedlove, 1988; Fishman and Breedlove, 1988). Androgen also increases SNB muscle fibers, motoneuron size and dendritic arborization in adult males (Wainman and Shipoundoff, 1941; Venable, 1966; Breedlove and Arnold, 1981; Kurz et al., 1986). The seasonal availability of hormones also effects SNB morphology in the white-footed mouse by increasing soma and nucleus size and dendritic arborization in animals exposed to long photoperiods compared to those exposed to short days (Forger and Breedlove, 1987).

In contrast, courtship neuromuscular systems have only been studied in non-mammalian vertebrates. Male African clawed frogs and midshipman fish vocalize during courtship and have larger vocalizing muscles compared to females (Kelley, 1986; Bass and Marchaterre, 1989b, Bass and Baker, 1990). The motor nuclei in these models contain more and larger motoneurons in males (Kelley, 1986; Bass, 1986; Bass and

Marchaterre, 1989a). Similarly, the vocal organ (syrinx) in the zebra finch and canary is larger and the nucleus controlling song production is greater in volume in males than females (Nottebohm and Arnold, 1976; DeVoogd and Nottebohm, 1981; Wade and Buhlman, 2000). Androgens can affect the ontogeny and maintenance of these neuromuscular systems. Prior to metamorphosis and in adulthood, androgens increase the number of axons of laryngeal muscle motoneurons and muscle fiber size in the African clawed frog (Sassoon and Kelley, 1986; Watson et al., 1993; Robertson et al., 1994). Androgen also masculinizes the neuromuscular system of the midshipman fish during development and in adulthood (Brantley et al., 1993). However, in adult songbirds, androgens modestly increase syrinx weight and muscle fiber size, and they have no effect on the volume of the hypoglossal nucleus or soma size and number (Wade and Buhlman, 2000).

Interestingly, neurons rostral to those in the sonic motor nucleus were labeled in the midshipman fish following biocytin injections to the sonic muscle (Bass et al., 1994). The authors suggest that these additional cells reflect transneuronal labeling, probably via gap junctions. Similar labeling has been reported in other sonic fish species (Ladich and Bass, 1996; Bass et al., 1996). In Study 2 we were surprised to find additional light labeling at T13-T15 and T11-T12 following TPN injections in some animals. The brown reaction product was in fact very light, which would be consistent with the biocytin being diluted as it passed retrogradely into neurons. It is possible that as in some sonic fish, the additional labeling represents transneuronal tracing, and that it identifies additional components of the copulatory circuit in male anoles. However, we hesitate to draw any conclusions without further investigation.

In the green anole lizard, neuromuscular systems controlling ***both*** courtship and copulation can be investigated. Males extend a throat fan (dewlap) to female during the courtship display, and several male-biased dimorphisms in this system have been detected. For example, the cartilage, muscle fibers, motoneurons and nerve responsible for dewlap extension are all increased in size in males compared to females (Wade, 1998; O'Bryant and Wade, 1999). Males also have more fibers than females overall and larger neuromuscular junctions on the ceratohyoid muscle (O'Bryant and Wade, personal communication). These structures appear to be stable in adulthood, as morphology does not change detectably with season or androgen manipulation. Mechanisms regulating sexual differentiation of the system are not yet known. With the development of this second system in the green anole, we now have a model organism in which neuromuscular systems regulating both courtship and copulation can be investigated. By comparing between these systems in the context of other models, we hope to eventually broaden the perspective on mechanisms regulating developmental organization and adult modulation of reproductive systems.

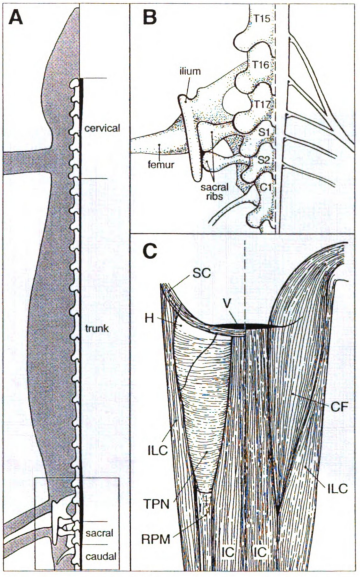

Figure 1. Vertebral, spinal and rostral tail muscle anatomy in the green anole lizard. In **A**, skeletal construction in relation to the body is illustrated. The boxed area is enlarged in **B** and on the left illustrates the sacral ribs articulating with the ilium, which in turn articulates with the femur. On the right side, the spinal cord and nerves are depicted. In **C,** a ventral view of the bilateral hemipene muscles are shown. The top muscle layers are depicted on the left side, and underlying structures are shown on the right. The transversus penis (TPN) muscle covers the hemipene (H), and the retractor penis magnus (RPM) runs from caudal vertebrae to insert at the posterior end of the hemipene. The caudifemoralis (CF) leg muscle resides primarily in the tail with the ischiocaudalis (IC) and the iliocaudalis (ILC) tail muscles. The sphincter cloacae (SC) muscle runs along the vent of the cloaca (V).

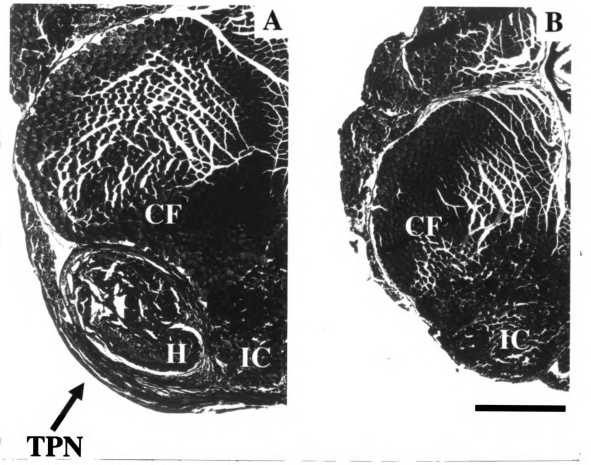

Figure 2. Cross-sections through the rostral tail in a male (**A**) and a female (**B**). The right side of each photograph corresponds to the midline of each section. Abbreviations as in Figure 1. Photos were taken with a Kodak DCS410 digital camera using Adobe Photoshop 5.0. Scale bar = 750μm.

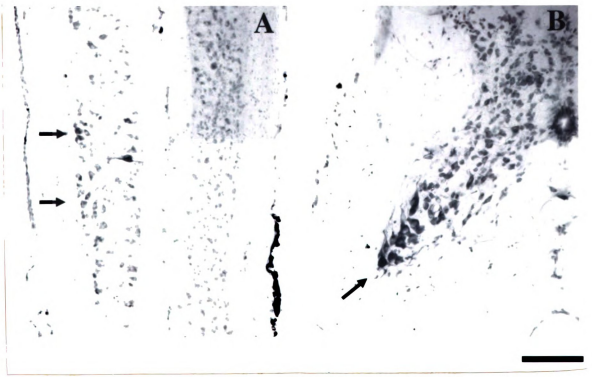

Figure 3. Horizontal (**A**) and coronal (**B**) sections through the last trunk segment (T17) of the spinal cord in a male containing biocytin-labeled motoneurons (indicated by the arrows) that project to the transversus penis muscle. The ink mark at sacral segment 1 can be seen in the lower right corner in **A**. Scale bar in **A**=300μm and in **B**=150μm.

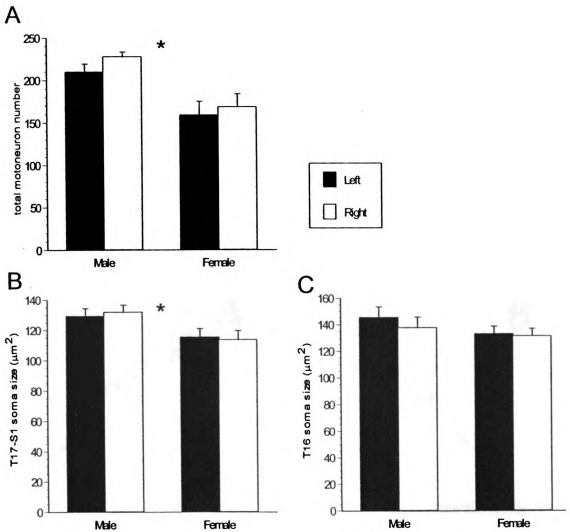

Figure 4. Total number (mean + S.E.; **A**) and soma size (mean + S.E.; **B**) of Nissl-stained motoneurons in T17-S1 in Study 3. Values indicated are double the number counted in every other section. As a control, soma size (mean + S.E.; **C**) was measured in T16 motoneurons. ***=males significantly greater than females.

Figure 5. Coronal section through the spinal cord at T17 in a male (**A**) and a female (**B**) showing the Nissl-stained motoneurons analyzed in Study 3 (identified with arrows). The right side of **A** and the left side of **B** correspond to the midline of each section. Photos were taken with a Kodak DCS410 digital camera using Adobe Photoshop 5.0. Scale bar = 200μm.

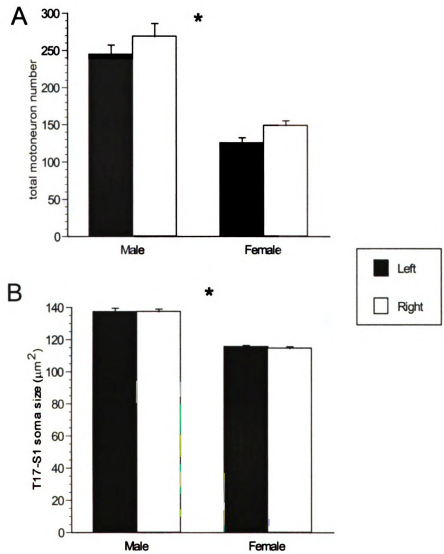

Figure 6. Cell counts (mean + S.E.; **A**) and soma size (mean + S.E.; **B**) of motoneurons in T17-S1 from Study 4. The left caudifemoralis of each individual was injected with biocytin to label the motoneurons projecting to that muscle. In every section, only biocytin-free, Nissl-stained cells were analyzed. *=males significantly greater than females.

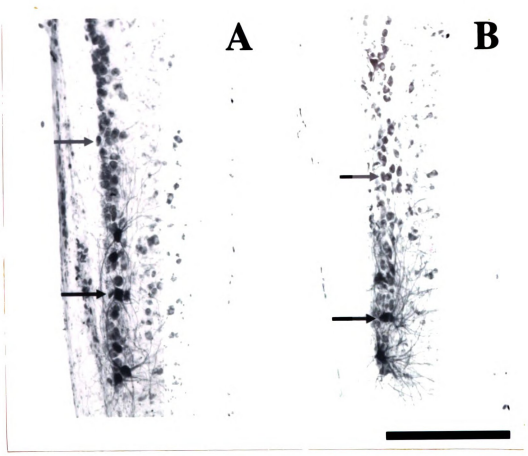

Figure 7. Horizontal sections through the left side of spinal cord segments T17-S1 in a male (**A**) and a female (**B**) with biocytin-labeled motoneurons projecting to the caudifemoralis muscle. The Nissl-stained (identified by the grey arrows) and biocytin-labeled cells (identified by the black arrows) were separately analyzed in Study 4. Photos were taken with a Kodak DCS410 digital camera using Adobe Photoshop 5.0. Scale bar = 300μm.

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