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Normally occurring intersexuality and testosterone induced plasticity in the copulatory system of adult leopard geckos

Melissa M. Holmes[a,*], Oliver Putz[b], David Crews[b], Juli Wade[a,c]

[a]Neuroscience Program, 108 Giltner Hall, Michigan State University, East Lansing, MI 48824-1101, USA
[b]Section of Integrative Biology, University of Texas at Austin, Austin, TX 78712, USA
[c]Departments of Psychology and Zoology, Michigan State University, East Lansing, MI 48824, USA

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Abstract

The copulatory neuromuscular system of lizards is highly sexually dimorphic. Adult males possess bilateral penises called hemipenes, which are independently controlled by two muscles, the retractor penis magnus (RPM) and transversus penis (TPN). These structures are not obvious in adult females. However, in adult female leopard geckos (*Eublepharis macularius*), testosterone induces hemipene growth. We investigated whether these structures develop de novo in adulthood or are histologically present as rudimentary structures in the female leopard gecko. We also investigated the extent of sexual dimorphisms and plasticity in the associated neuromuscular components. To do this, we compared copulatory morphology (sizes of hemipenes, RPM and TPN muscle fibers, and associated motoneurons, as well as motoneuron and RPM fiber number) in adult females treated with testosterone, control females, and control males. All of the geckos possessed hemipenes, RPMs and TPNs, but these structures were indeed vestigial in control females. Testosterone induced striking increases in hemipene and copulatory muscle fiber size in females, but not to levels equivalent to control males. In parallel, males with increased levels of androgenic activity had larger hemipenes, suggesting naturally occurring steroid-induced plasticity. Copulatory motoneurons were not sexually dimorphic in size or number, and these measures did not respond to testosterone. The data demonstrate that the copulatory system of leopard geckos, in which gonadal sex is determined by egg incubation temperature, differs from that of many species (both reptilian and mammalian) with genotypic sex determination. Indeed, the system is remarkable in that adult females have normally occurring intersex characteristics and they exhibit substantial steroid-induced morphological plasticity in adulthood.

Keywords: Androgen; Intersex; Leopard gecko; Sex difference; Sexual dimorphism; Temperature dependent sex determination; Testosterone

Introduction

Comparative studies involving neuromuscular systems offer a powerful means of elucidating mechanisms regulating both sexual differentiation of and adult plasticity in the nervous system. The copulatory neuromuscular system is particularly useful in this regard. In mammals this system shows a striking degree of both sexual differentiation and adult plasticity. A clitoris forms instead of a penis in developing females, and the motoneurons and muscles of the masculine copulatory neuromuscular system regress (Breedlove et al., 2002). Survival and further development of the copulatory neuroeffectors in males is dependent on androgens, and the sensitivity to androgens remains into adulthood (Breedlove et al., 2002). Seasonal increases in testosterone (T) result in increased motoneuron soma and muscle fiber size and, in parallel, stimulate copulatory behavior (Forger and Breedlove, 1987; Hegstrom et al., 2002). In contrast, in females androgens cannot resurrect the penis, muscles, or motoneurons in adulthood (e.g., Breedlove and Arnold, 1983; Tobin and Joubert, 1991).

Similar to mammals, lizards possess a sexually dimorphic copulatory neuromuscular system (Holmes and Wade, 2004a; Ruiz and Wade, 2002). Male lizards possess two

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* Corresponding author. Fax: +1 517 432 2744.
E-mail address: holmes22@msu.edu (M.M. Holmes).

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intromittent copulatory organs as well as associated muscles; these structures develop in embryos of both sexes and then regress in females prior to or around hatching (Raynaud and Pieau, 1985). The intromittent organs are called hemipenes, each controlled by two muscles that receive ipsilateral projections from motoneurons in the spinal cord (Arnold, 1984; Holmes and Wade, 2004a). Green anoles (*Anolis carolinensis*), which exhibit male heterogametic genotypic sex determination (Adkins-Regan, 1981), are the only reptilian species in which sexually dimorphic neuromuscular systems have been investigated in detail. In this species, adult females lack all components of the male copulatory neuromuscular system (Holmes and Wade, 2004a; Ruiz and Wade, 2002). Similar to mammals, regression of the hemipenes and associated muscles cannot be reversed by T treatment in juvenile females (Lovern et al., 2004), although T stimulates growth of the hemipenes and muscle fibers in juvenile and adult males housed under environmental conditions conducive to breeding (Holmes and Wade, 2004b; Lovern et al., 2004).

Interestingly, unlike other vertebrates, adult female leopard geckos (*Eublepharis macularius*) appear capable of growing penile structures when treated with T (Rhen et al., 1999). It was unclear, however, whether the associated neuromuscular structures would respond in parallel. In addition, while testicular hormones in a variety of species are responsible for masculine organization, and sometimes adult maintenance, of regions of the central nervous system and muscles that control reproductive function, evidence from some mammalian and avian systems indicates roles for the direct action of sex-linked genes (reviewed in Crews, 1996; Wade and Arnold, 2004). As such, it was also of great interest to determine the degree of sexual differentiation and response to adult T in the copulatory neuromuscular system in a species without genetic sex determination. Leopard geckos lack sex chromosomes and exhibit temperature dependent sex determination; the gonadal and behavioral sex of the animal depends on the temperature at which its egg incubated (Gutzke and Crews, 1988; Rhen and Crews, 1999; Viets et al., 1993). We first confirmed the location of their copulatory motoneurons and then investigated whether the copulatory organs and the associated neuromuscular components develop in females de novo in adulthood, or whether T induces plasticity in structures that already exist.

Materials and methods

Animal care

Freshly laid leopard gecko eggs were incubated in moist vermiculite (1.5 water: 1 vermiculite) in temperature-controlled incubators (accuracy ±0.1°C). Both male and female geckos were used; males were incubated at 32.5°C (which produces approximately 75% males; Viets et al., 1993) and females were incubated at 30°C (which produces approximately 75% females; Viets et al., 1993). After hatching, animals were raised in isolation in propylene containers (30 × 12 × 6 cm) containing a water dish and shelter and fed live vitamin supplement-dusted crickets daily. During weeks 1–10 posthatching, juveniles were raised in environmental chambers set to a constant temperature of 30°C and a light–dark cycle of 14:10. Subsequently, chambers were maintained on a temperature cycle of 30:18°C corresponding to a 14:10 light–dark cycle. The diet from 10 weeks onward consisted of water and mealworms dusted with vitamin supplements fed three times per week. Procedures adhered to institutional and NIH guidelines for animal use and care.

Confirmation of motoneuron location

The copulatory neuromuscular system is bilateral in lizards. The two major pairs of muscles controlling hemipene function are the transversus penis (TPN), which wrap around each hemipene and mediate its eversion, and the retractor penis magnus (RPM), which attach to the base of each hemipene and mediate retraction of the organs. The hemipenes, TPNs, and RPMs are all located in the rostral tail (see diagrams in Arnold, 1984; Ruiz and Wade, 2002), and in green anoles the corresponding motoneurons are found in the pelvic region of the spinal cord, interdigitated with motoneurons projecting to the caudifemoralis (CF; a leg muscle) and the cloacal sphincter (Holmes and Wade, 2004a; Ruiz and Wade, 2002). Prior to the commencement of the experiment investigating sexual dimorphisms and adult plasticity, confirmation of the location of motoneurons projecting to copulatory muscles in leopard geckos was performed as in Holmes and Wade (2004a). Briefly, four adult male leopard geckos were anesthetized and incisions were made in the ventral surface of the tail. Two males received a unilateral injection of Fast Blue (Illing Plastics, Bergfeld, Germany; 1.0 μl at 3% in 0.9% saline) into the RPM and two males received TPN injections in the same manner. To decrease possible contamination, excess tracer was removed with a cotton swab and gel foam prior to suturing the incision with silk.

Five days following the injections, lizards were anesthetized and perfused with 0.1 M phosphate-buffered saline (PBS; pH 7.4) and 4% paraformaldehyde in PBS. Spinal segments were marked with India ink (brushed on each dorsal spinal nerve root) to facilitate identification of individual segments. Cords and rostral tails (to confirm injection site) were extracted (three caudal-most trunk segments and sacral segments 1 and 2), embedded in gelatin, postfixed in 4% paraformaldehyde for 2.5 h, then transferred to 20% sucrose in PBS overnight. All remaining histology and analyses were performed exactly as described in Holmes and Wade (2004a). As in the green anole, all fast blue-labeled motoneurons were located in the last trunk and first sacral segment, and no labeled motoneurons were seen in any other spinal segment (Fig. 1). Because geckos usually