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

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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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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 $\pm 0.1^{\circ}$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

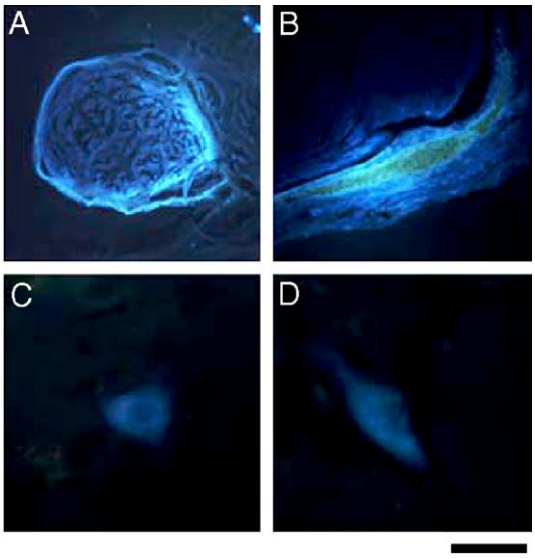

Fig. 1. Photomicrographs of muscles and motoneurons from adult male leopard geckos labeled with Fast Blue. (A) FB-injected RPM and (C) corresponding FB+ motoneuron in spinal segment T18. (B) FB-injected TPN and (D) corresponding FB+ motoneuron in spinal segment T18. At the level of this TPN photomicrograph, many of the fibers run medial to lateral, perpendicular to the RPM fibers (see Materials and methods). Photomicrographs A and C were from one male; B and D were taken from a different male. Scale bar = 600 μm (A and B) and 20 μm (C and D).

have 18 trunk segments (Hoffstetter and Gasc, 1969; Romer, 1956), as compared to 17 in the green anole (Ruiz and Wade, 2002), labeled cells were in segments trunk 18 and sacral 1 (T18-S1).

Experimental treatment and tissue collection

Adult animals (1.5–3 years of age) received a unilateral subcutaneous Silastic implant (20 mm × 1.47 mm ID × 1.95 mm OD) filled with either T (Steraloids Inc., Wilton, NH; n = 8 females) or nothing (n = 8 females and n = 8 males) as described previously (Rhen and Crews, 1999; Rhen et al., 1999). Four weeks after surgery, animals were measured snout to vent (SVL) and anesthetized prior to perfusion with PBS (pH 7.4) and 4% paraformaldehyde in PBS. All tissue was collected as in Holmes and Wade (2004b). Immediately prior to perfusion, gentle pressure was applied to the tail at the base of one randomly selected hemipene per animal to test whether it was evertable. Spinal column, kidneys, gonads, and rostral tail (containing hemipenes, TPNs, and RPMs) were extracted and postfixed in 4% paraformaldehyde for 1 week. Spinal cords were extracted and marked as above, embedded in gelatin, and sunk overnight in 20% sucrose in 4% paraformaldehyde. Spinal cords were then sectioned frozen in the coronal plane at 30 μm; every other section was mounted out of 9:1 dH2O–0.1 M PBS onto gelatin-coated slides. Spinal cord tissue was then stained with thionin, dehydrated and coverslipped using Permount.

Kidneys, gonads, and tails were then transferred to Bouin's fixative for 1 week. Only the ventral half of the tail was maintained in order to provide better exposure of the tissues of interest to fixative and paraffin (see below). The tissues were then soaked in 70% ethanol overnight, dehydrated, cleared in xylene, and embedded in paraffin. These tissues were then sectioned at 10 μm; tail sections were stained with the trichrome method, and kidneys and gonads were stained with hematoxylin and eosin.

Morphological measurements and statistical analyses

All measurements were conducted without knowledge of sex or treatment. Motoneurons were analyzed as in Holmes and Wade (2004b) and Ruiz and Wade (2002). The soma size of 20 randomly selected motoneurons was measured through the rostral–caudal extent of the spinal segments of interest using Scion Image (Scion Corp., PC-based software adapted from NIH Image). For copulatory motoneurons, cells were measured in segments T18-S1 on each of the right and left sides. Soma size was also measured on each side of the cord in spinal segments T16-17 as a control; motoneurons rostral to the last trunk segment (T17 in green anoles and T18 in leopard geckos) do not project to the TPN or RPM (Holmes and Wade, 2004a; Ruiz and Wade, 2002; present experiment). An estimate of total motoneuron number was obtained separately for spinal segments T16-17 and T18-S1; motoneurons were counted in alternate sections for each side of the spinal cord, totals were multiplied by two, and then the left and right counts were averaged to provide an estimate for one side of the spinal cord. For T16-17 counts, all cells were counted in tissue sections between the rostral-most extent of the T16 nerve and the caudal-most extent of the T17 nerve. Similarly, T18-S1 counts were from sections between the rostral-most extent of the T18 nerve and the caudal-most extent of the S1 nerve. There were no statistically significant differences among the three groups of animals in the number of sections in which motoneurons were counted (data not shown); therefore, spinal cord segment length was not a factor in overall cell number estimates. Each motoneuron was counted only if its nucleus both came into focus and disappeared within a given section (the physical dissector technique) to avoid double counting of cells across sections (Gunderson, 1986; Ruiz and Wade, 2002).

The cross-sectional area of 25 randomly selected RPM fibers was measured per side (Holmes and Wade, 2004b), and the number of muscle fibers was counted in a single cross-section in the top third of the muscle. Unlike other lizards, including the green anole, in which the TPN fibers wrap around the hemipenes from medial to lateral, in geckos the majority of these muscle fibers are arranged in an approximate longitudinal orientation (see Arnold, 1984; Ruiz and Wade, 2002 for schematics). Thus, sufficient TPN fibers that obviously ran rostral to caudal could be measured as for the RPM. However, because a portion run medial to

lateral across part of the hemipene, a total number could not be estimated with confidence. As a control for the specificity of effects on copulatory muscles, CF fiber size was measured in the same manner as the RPM. Hemipene size was estimated by measuring the cross-sectional area of each hemipene in 15 sections, approximately 50 μm apart through the rostral ~750 μm of the structure. Finally, renal sex segment epithelial cell height was measured in four cells from each of four randomly selected tubules (resulting in 16 measures), and gonads were evaluated for spermatogenesis (in males) and degree of follicular development (in females). In lizards, renal "sex segments" perform functions similar to the mammalian prostate and enlarge in response to androgen, thus providing a bioassay for T exposure (Cueller et al., 1972; Winkler and Wade, 1998).

For all animals, mean size was calculated separately for each side of the body for all tissues (except kidneys and gonads). 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 T18-S1 and T16-17; 50 muscle fibers for RPM, TPN, and CF; and 30 sections for hemipenes).

The data were not normally distributed and transforming them did not produce a normal distribution. Thus, non-parametric statistics were employed for all analyses. Each measure was analyzed across the three treatment groups using Kruskal–Wallis tests. When significant group differences were detected ($P < 0.05$), pairwise comparisons were conducted using Mann–Whitney tests. As the direction of the effects was already known, these tests were one tailed. P values were considered statistically significant if less than 0.033 (Bonferroni correction; 0.10 divided by three possible pairwise comparisons).

Results

General/control measures

Renal sex segment cell height differed among the three experimental groups ($H = 13.21$, $P = 0.001$; Fig. 2). T-treated females had significantly larger cells compared to both males ($P = 0.01$) and control females ($P < 0.001$), whereas males and control females did not differ from each other ($P = 0.17$). However, a bimodal distribution was detected among the males on this measure; four had large cells (approximately equivalent to T-treated females) and four had small cells (approximately equivalent to control females; Fig. 2; see below). All males had testes and all females, regardless of treatment, had ovaries with at least one yolking follicle; there was no indication of testicular tissue in any female. Condition of the testes and vasa deferentia in males matched renal sex segment development; only the four males with large kidney cells showed evidence of mature sperm.

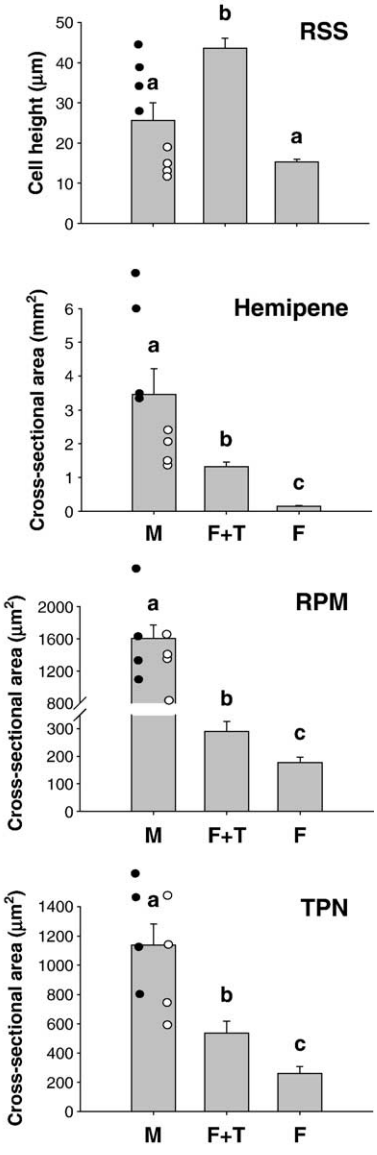

Fig. 2. Mean (±SEM) values for renal sex segment (RSS) cell height, hemipene cross-sectional area, RPM muscle fiber cross-sectional area, and TPN muscle fiber cross-sectional area. Treatment groups included control males (M), females with a testosterone implant (F + T), and control females (F). Open circles represent individual data points for males with smaller renal sex segment cells and closed circles represent males with larger renal sex segment cells. For all variables except renal sex segment cell height, all three groups were significantly different from each other. For renal sex segment cell height, F + T animals were significantly larger than both other groups.

SVL was greater in males than both groups of females as detected by the Kruskal–Wallis test ($H = 7.23$, $P = 0.03$). However, differences between the individual groups all failed to reach statistical significance ($P \geq 0.04$; Bonferroni correction; Table 1). Importantly, correcting for body size (by dividing the variable of interest by SVL for each individual) did not minimize the statistical significance of any effects reported below. In addition, no significant differences were detected among groups for the measures of control tissues (Table 1): not CF muscle

Table 1
Mean (±SEM) count and size estimates for copulatory (T18-S1) and non-copulatory (T16-17) motoneurons, a muscle involved in copulation (RPM; count only), and a control muscle (CF; size only)

	Male	Female + T	Female
T18-S1 count	199.81	210.81	189.44
	(10.09)	(12.88)	(5.29)
T18-S1 soma size (μm^2)	414.98	379.90	370.00
	(27.27)	(23.33)	(30.63)
T16-17 count	189.29	197.38	198.00
	(11.8)	(10.46)	(18.71)
T16-17 soma size (μm^2)	402.01	392.5	369.39
	(28.51)	(19.59)	(27.51)
RPM fiber count	407.63	493.64	436.75
	(56.73)	(39.79)	(30.95)
CF fiber size (μm^2)	2667.59	2890.76	3045.32
	(184.33)	(201.57)	(203.31)
SVL (mm)	12.86	12.05	12.01
	(0.15)	(0.24)	(0.24)

Mean (±SEM) snout to vent length (SVL) is also shown. No statistically significant effects were detected with pairwise comparisons on these variables.

fiber size ($H = 1.72$, $P = 0.42$), nor motoneuron number ($H = 0.48$, $P = 0.79$) or soma size ($H = 0.50$, $P = 0.78$) in spinal segments T16-17, rostral to those containing RPM- and TPN-projecting cells.

Copulatory structures

All animals possessed hemipenes as well as both associated muscles, the RPM and TPN, but hemipenes could be everted only in males and T-treated females. Hemipene cross-sectional area differed markedly among the three groups ($H = 18.74$, $P < 0.001$; Figs. 2 and 3). Males had larger hemipenes than both female groups (both $P < 0.007$), and T treatment increased hemipene size in females ($P < 0.001$). RPM and TPN fiber size were both significantly different among groups (RPM: $H = 17.10$, $P < 0.001$; TPN: $H = 15.61$, $P < 0.001$; Figs. 2 and 3). RPM and TPN fibers were larger in males than in both groups of females (all $P < 0.004$), and T-treated females had larger RPM and TPN fibers than control females (both $P < 0.03$). RPM fiber number was equivalent across the groups ($H = 1.77$, $P = 0.41$; Table 1). Finally, neither the number of motoneurons ($H = 1.38$, $P = 0.50$; Table 1) nor the soma size of cells in T18-S1 ($H = 0.90$, $P = 0.64$; Table 1) was affected by sex or T manipulation in females.

Discussion

The present data demonstrate that adult female leopard geckos possess hemipenes as well as the complete neuromuscular system required for masculine copulatory behavior. Furthermore, the copulatory muscles and hemipenes are remarkably sensitive to androgens in adulthood; muscle fibers approximately doubled in size and hemipene cross-sectional area increased by an order of magnitude in females treated with T. While T commonly enhances these types of structures in adult male mammals, the structures are absent in adult females (reviewed in Breedlove et al., 2002). Therefore, the normal possession of independent, discrete vestigial masculine copulatory organs and their neuromuscular effectors in adult breeding female leopard geckos is an apparently rare phenomenon. Indeed, in the only other lizard species in which these structures have been investigated in detail, the green anole, males possess all aspects of the system while females do not (Ruiz and Wade, 2002). These neuromuscular structures are morphologically sensitive to T in male but not female anoles (the system cannot be resurrected; Holmes and Wade, 2004b; Lovern et al., 2004).

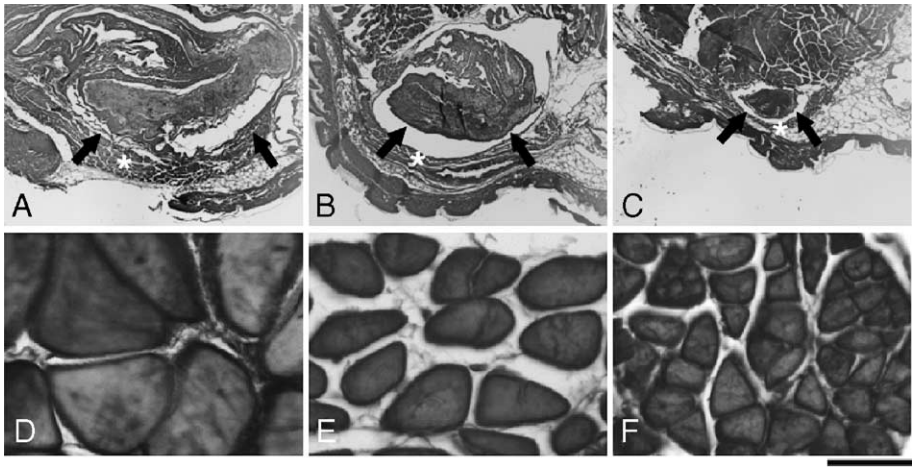

Fig. 3. Photomicrographs of cross-sections through the hemipenes (A–C) and TPN muscle fibers (D–F). A and D are from a control male, B and E are from a female with a testosterone implant, and C and F are from a control female. Black arrows point to the ventral edge of each hemipene, adjacent to the TPN muscle (labeled with white asterisk). In panel A, the dorsal surface of the hemipene is just above the top of the field of view. Scale bar = 1 mm (A, B, C) and 35 μm (D, E, F).

Why are the hemipenes, muscles, and motoneurons maintained in adult female leopard geckos? We know of no function the hemipenes could serve in female reproductive or other behaviors. And, despite having all components of the system (including hemipenes, muscles, and an equivalent number of motoneurons with comparable soma size), the hemipenes can only be everted following T treatment (Rhen et al., 1999; present study), not in normal females that have lower T. Thus, the most likely explanation for maintenance of the system in females is that it is a by-product of a developmental event required for both sexes that happens to coincide with the critical period for sexual differentiation of copulatory structures. While it is not yet known whether sexual differentiation of this system is dependent on androgens, if the mechanism of differentiation parallels the mammalian copulatory system (Breedlove et al., 2002), one possibility is that developmental androgens (perhaps from an adrenal source) are high enough to maintain the neuromuscular system in both sexes. Leopard gecko hemipenes do possess androgen receptors in adulthood (Endo and Park, 2003; Rhen and Crews, 2001), although distribution of this protein in the copulatory muscles and motoneurons has not yet been investigated.

In sexually dimorphic neuromuscular systems, steroid-induced increases in morphology usually occur in concert with increased behavioral expression. This association between structure and function exists in the dimorphic systems that mediate courtship vocalizations in fish, frogs, and songbirds (Brantley et al., 1993; Kelley, 1986; Nottebohm, 1981), as well as the copulatory neuromuscular system in lizards and rodents (Breedlove and Arnold, 1981; Forger and Breedlove, 1987; Hegstrom et al., 2002; Holmes and Wade, 2004b; but see Breedlove, 1997). Indeed, the relationship between male hemipene size and androgen levels in the present experiment, as well as the fact that T increases mounting behavior in males (Rhen and Crews, 1999), suggests that the hormone also has parallel effects on behavior and morphology in male leopard geckos. However, while high levels of T for long periods (e.g., 4 weeks) has substantial masculinizing effects on hemipenes and copulatory muscles in adult females, the same treatment does not result in increased expression of masculine reproductive behaviors by these females (Rhen and Crews, 1999). Similar hormone treatment actually results in decreased receptive behaviors and attractiveness to males (although lower doses of T facilitate female-typical sexual behaviors; Rhen et al., 1999). It is possible that incomplete masculinization of morphology and/or behavior might be attributed to an insufficient dose of T, but this seems unlikely as implants of this size in adult females produce T levels equivalent to breeding males (Flores and Crews, 1995; Rhen et al., 1999). It is also possible that T would have a greater effect in females from a male-biased incubation temperature as temperature can organize traits independently of sex hormones in this species (Flores and Crews, 1995; Tousignant and Crews, 1995). The incubation temperatures in the present study were selected in order to maximize detection of potential sex differences, but this issue warrants further investigation.

The classical view of sexual differentiation in mammals is that a genetic signal on the Y chromosome triggers testicular differentiation, and the secretion of hormones from the testes initiates development of masculine secondary sex characteristics, including genitalia. This cascade occurs in a sex-specific and non-reversible manner with the absence of a Y chromosome and consequently low levels of T being responsible for the development of many feminine characteristics (Wade and Arnold, 2004). While the genetic mechanisms influencing gonadal differentiation are not clear in birds (Smith and Sinclair, 2004), a similar pattern seems to occur for some aspects of sexual differentiation with the homogametic sex (male ZZ) being "default" (Adkins-Regan, 1981). Among reptilian species, examples of both XX/XY and ZZ/ZW genotypic sex determination exist, as well as those like leopard geckos that lack sex chromosomes altogether and utilize temperature dependent sex determination.

The fact that T can induce hemipene growth in leopard gecko females from a variety of incubation temperatures (Rhen et al., 1999) suggests that it is not incubation temperature per se that mediates the incomplete differentiation. Rather, it is possible that the degrees of early organization of gender-related characteristics, as well as adult plasticity, are mediated at least in part by the mode of sex determination. Sexual differentiation of the copulatory muscles and hemipenes in green anoles (XX/XY) is complete by hatching, not reversible by T in juveniles, and these structures are less plastic in adulthood compared to leopard geckos (Holmes and Wade, 2004b; Lovern et al., 2004; present data). In contrast, some evidence for incomplete differentiation of hemipenes exists in colubrid snakes with ZZ/ZW sex determination (Hardy, 1970; Hoge et al., 1959). However, even within this group of reptiles, females from the vast majority of species investigated do not have hemipenes (Hardy, 1970). Taken together, it is interesting to speculate that environmental sex determination and perhaps female heterogamety may be related to less organization and increased potential for morphological plasticity. Consistent with this idea is the striking plasticity often seen in the adult songbird brain (reviewed in Brenowitz, 2004; Tramontin and Brenowitz, 2000). Of course it must be acknowledged that at present we have complete information on the copulatory system of only one reptilian species with genetic and one with environmental sex determination (and no example of a ZZ/ZW species). To test hypotheses regarding the role of genetic influences, and sex chromosomes in particular, it will be critical to integrate information from more reptilian species with divergent methods of sex determination, as well as from additional avian and mammalian species.

Acknowledgments

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