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Abstract.—**In this study we investigate hemipenial variation through ontogeny by preparing specimens of known-aged individuals from captive-bred Plains Gartersnakes, *Thamnophis radix*, descended from a wild population in northern Illinois, USA. We examined males at two different ages (215–254 days, $N = 9$) and (829–867 days, $N = 12$) to compare both juvenile and adult morphologies. Hemipenis length increased isometrically with tail length, and there were no significant differences detected between right and left hemipenis length or width. In addition, this study is the first to explore variation in hemipenial morphology within and among litters. We found significant litter effects on hemipenis length, on the elevation (but not the slope) of the relationship between hemipenis length and tail length, and on number of basal hooks, suggesting a possible genetic basis to these characteristics. These results highlight the importance of examining multiple males through ontogeny, as well as reporting body-size measurements for all specimens, to obtain an accurate representation of the hemipenis morphology of a species for comparative ecological, taxonomic, and evolutionary studies.**

Among snake species, variations in male reproductive organs (i.e., hemipenes) are considerable, encompassing differences in shape, dimensions, and ornamentation (Dowling and Savage, 1960; Zaher, 1999). The number of spines, calyces, hooks, and length and width of hemipenes and hemipenial lobes, along with many other characters, provide distinctions within genera and across taxonomic groups (Myers, 1974; Schargel and Castoe, 2003; Schargel et al., 2005; Jadin et al., 2010b). Hemipenial characters are evolutionarily plastic and suggested to be under weaker natural selection compared with external morphological features (Dowling, 1967; Jadin and Parkhill, 2011).

Understanding the evolutionary relationships and reproductive behavior of snakes is enhanced by examining the morphological features of male genitalia and comparing among taxa (Keogh, 1999; King et al., 2009; Jadin et al., 2010a). Although many studies have compared hemipenial structures across taxa, and to a lesser extent within a taxon (Zaher and Prudente, 1999; Shine et al., 2000), few ontogenetic analyses of hemipenis morphology exist (Raxworthy and Nussbaum, 2006). As a consequence, how hemipenis morphological complexity and ornamentation reflect ontogeny and the onset of sexual maturity remain unknown.

Our objective was to investigate variation in hemipenial size and morphological characters through ontogeny. We took the novel approach of comparing hemipenial morphology between siblings differing in age in the Plains Gartersnake, *Thamnophis radix*. We choose this species because it possesses highly ornamented hemipenes, possibly resulting from complex mating behavior (King et al., 2009). We focused on hemipenial characters (e.g., length and width of hemipenis, number of spines) that may be important in copulatory behavior. Furthermore, we included tail length (TaL) as a measure of overall size. This approach allowed us to test the prediction that hemipenis length shows negative allometry with body size in this species (King et al., 2009). Finally, we tested for differences among litters in hemipenial morphology because such a difference might suggest a genetic basis to hemipenis morphology (Brodie and Garland, 1993).

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Materials and Methods

Morphological Data.—Study specimens consisted of 21 first- and second-generation captive-bred male Plains Gartersnakes descended from five wild-caught females from a site in DeKalb County, Illinois (Stanford and King, 2004). Offspring represented six sets of singly sired full-siblings. Several consanguineous matings during captive propagation resulted in estimated relatedness ranging from 0.50 to 0.63 among full-siblings and from 0.06 to 0.22 among nonsiblings (estimated using Pedigraph; Garbe and Da, 2008). Snakes were euthanized at 215–254 or 829–867 days (157–257 and 374–464 mm snout–vent length [SVL], respectively). Based on data from the population from which study animals were derived (Stanford and King, 2004), these snakes correspond to juvenile ($N = 9$) and adult ($N = 12$) individuals, respectively. Ages of snakes within litters varied by a maximum of 14 days. Specimens were deposited at the Amphibian and Reptile Diversity Research Center at the University of Texas at Arlington (UTA R-59188–208).

After euthanasia, hemipenes were everted, filled with warmed petroleum jelly by using a syringe fitted with a ball-tipped gavage needle, and tied using fine thread. Specimens were fixed in 10% formalin and stored in 70% ethanol. We examined the left and right hemipenes in all specimens except for cases where one or both of the hemipenes were damaged or not fully everted ($N = 5$). For each snake, we measured SVL, TaL, length of each hemipenis (HL) starting at the cloaca to the end of the sulcus spermaticus, and width of each hemipenis (HW) going across directly above the most anterior hook (Fig. 1). We also recorded number of basal hooks on each hemipenial lobe (NHL) and length of largest basal hook (LLH) for the older age class. All measurements were collected using a dissecting scope or meter stick.

Statistical Analyses.—Paired *t*-tests were used to compare right and left hemipenes for HL, HW, NHL, and LLH for adults. Test-wise α values were adjusted using the sequential Bonferroni approach to maintain an experiment-wise $\alpha = 0.05$ (Rice, 1989). When no differences were found, means (length, width, hook length) or sums (number of hooks) were calculated for subsequent analyses.

Snout–vent length and TaL were strongly correlated with each other (see Results), but TaL was more strongly correlated with HL. Thus, we used analysis of covariance with TaL as a covariate and litter as a factor to characterize the ontogeny of

FIG. 1. Sulcate view of left and right hemipenes of a young age class individual (A, UTA R-59198) and three large age-class individuals (B, UTA R-59199; C, UTA R-59191; D, UTA R-59203). Specimens UTA R-59198 (A) and UTA R-59199 (B) are direct siblings but the rest are not, illustrating the drastic difference between age classes and among litters but the lack of difference between left and right hemipenes. Arrows point to hooks on the hemipenial base, and length and width bars on the right side illustrate how we measured the hemipenes. Length and width bar accurately measures the right hemipenis of UTA R-59203 (D).

HL. Both HL and TaL were transformed by taking natural logarithms before analysis to better meet assumptions of analysis of covariance and to provide allometric coefficients relating HL and TaL. Statistical significance was assessed at $\alpha = 0.05$, and all analyses were conducted using SPSS 15.0.

Tests of litter effects were restricted to adult males (835–857 days of age). We used analysis of covariance to test for litter effects on hemipenis and hook dimensions. We used analysis of variance to test for litter effects on number of hooks.

RESULTS

Snout–vent length (range [in millimeters] = 157–464, $\bar{x} = 326.81$) and TaL (range = 49–142 mm, $\bar{x} = 97.24$) were correlated strongly with each other ($r = 0.97$, $P < 0.001$, $N = 21$), and both were correlated positively with average HL (range = 5.2–23.5 mm, $\bar{x} = 15.12$). The correlation was slightly stronger for TaL ($r = 0.93$, $P < 0.001$, $N = 20$) than for SVL ($r = 0.89$, $P < 0.001$, $N = 20$). Hemipenis length and HW (range = 2.75–4.55 mm, $\bar{x} = 3.65$) were uncorrelated ($t = 0.05$, $P = 0.875$, $N = 11$) as were LLH (range = 1.9–4.35 mm, $\bar{x} = 3.08$) and HL ($t = 0.32$, $P = 0.403$, $N = 9$). Curiously, the LLH was negatively correlated with average HW ($r = -0.84$, $P = 0.009$, $N = 8$). After sequential Bonferroni adjustment, paired t-tests revealed no differences between right and left hemipenis in length ($t = 0.68$, df = 11, $P = 0.508$), width ($t = 1.39$, df = 10, $P = 0.194$), number of hooks ($t = 2.39$, df = 10, $P = 0.038$), or LLH ($t = 0.28$, df = 8, $P = 0.789$).

The allometric coefficient relating ln(HL) to ln(TaL) equaled 1.09 ($F_{2,10} = 18.41$, $P < 0.001$) and did not differ from 1 (95% confidence interval = 0.91, 1.27), indicating that HL increases isometrically with TaL (Fig. 2). The relationship of HL to TaL did not differ in slope among litters ($F_{2,8} = 4.35$, $P = 0.053$) but did differ in intercept (i.e., elevation; $F_{2,10} = 18.41$, $P < 0.001$).

Within the younger age class, we did not detect more than one measureable spine, likely homologous to hooks in the older class, even though HL was generally at least half the length of the older age class. In addition, bilobation of the hemipenis apex was underdeveloped and not much wider than the pedicel (Fig. 1).

Among adult males, there was no litter-by-tail length interaction ($F_{3,4} = 0.45$, $P = 0.729$), but there were significant effects of litter ($F_{3,7} = 8.72$, $P = 0.009$) and TaL ($F_{1,7} = 6.18$, $P = 0.042$) on HL. There were no significant relationships among these variables and HW ($P > 0.390$) or largest hook length ($P > 0.160$). Total number of hooks (range = 3–5, $\bar{x} = 4.27$) differed significantly among litters ($F_{3,7} = 5.53$, $P = 0.029$).

DISCUSSION

Ontogeny and Hemipenial Morphology.—We found marked ontogenetic changes in hemipenis morphology. Snakes in the younger age class, for the most part, lacked measurable spines and showed minimal bilobation at the hemipenis apex (Fig. 1A). Furthermore, some individuals in the older class had

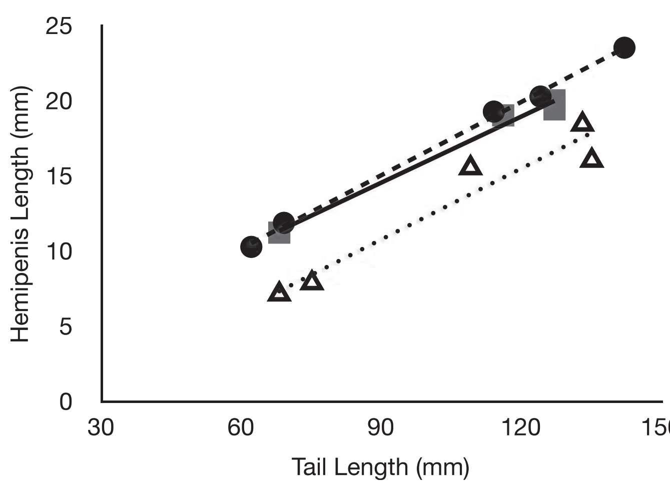

FIG. 2. Isometric relationship between HL and TaL. Symbols and separate lines represent three sets of full siblings (triangles associated with dotted line, closed circles with dashed line, and squares with solid line). The relationship between ln(HL) and ln(TaL) did not differ in slope ($F_{2,8} = 4.5$, $P = 0.053$) but did differ in elevation among litters ($F_{2,10} = 18.41$, $P < 0.001$).

apparently incompletely developed hemipenes (Fig. 1C), even though the older age class was similar in size to sexually mature males in nature and males in this population reach reproductive maturity at 2–3 yr of age (Stanford and King, 2004). These results highlight that accurately characterizing hemipenis structure and morphology (e.g., for taxonomic purposes) requires looking at fully mature adults, because many hemipenial features may not be developed in younger individuals. It is noteworthy that if the evolutionary trajectory of hemipenis morphology parallels this ontogenetic trajectory, we might conclude that a simple and subcylindrical hemipenis is ancestral to a bilobed hemipenis.

Hemipenis Allometry.—We found a strong positive correlation between body size and HL in *T. radix*. However, in contrast to other aspects of snake morphology (e.g., head dimensions, TaL; Arnold and Peterson, 1989), scaling was isometric rather than allometric (Fig. 2). This observation contradicts the prediction by King et al. (2009) that HL should exhibit negative allometric scaling as a strategy for small males to remain in copula longer, thus increasing their success at sperm competition. Hemipenis width was correlated negatively with LLH, suggesting that males with reduced HW may compensate with enlarged hooks to remain in copulation longer.

Asymmetry between Right and Left Hemipenes.—Shine et al. (2000) hypothesized that the right hemipenis of snakes is larger than that of the left hemipenis due to increased right testes size and therefore a preference for the right hemipenis during copulation. Our study did not support a size bias, and we found that neither HL nor HW differed significantly between left and right hemipenes. Neither this study nor Shine et al. (2000) found significant differences in HL between right and left hemipenes; however, Shine et al. (2000) found a slight (ca. 0.1 mm) but significant increased width of the right hemipenis compared with the left hemipenis.

Hemipenial Differences among Litters.—Despite our relatively low sample size, we found significant differences among litters in HL and number of hooks. These differences among litters may reflect quantitative genetic variation in those traits and hence an ability for hemipenis morphology to evolve in response to natural and sexual selection, possibly providing a fast-evolving morphological characteristic for evolutionary studies. Larger sample sizes would be needed to estimate the magnitude of heritability or genetic correlation (Brodie and Garland, 1993). Nevertheless, these results demonstrate the importance of examining hemipenes of multiple specimens to obtain an accurate representation of the morphological variation within a species for both taxonomic and ecological studies.

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