

Sexual dimorphism in the second-to-fourth digit length ratio in green anoles, *Anolis carolinensis* (Squamata: Polychrotidae), from the southeastern United States

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Abstract: Digit length ratios are organized during embryonic development and may show sexual dimorphism related to steroid exposure. The second-to-fourth digit length ratio (2D:4D) has received the most attention. In the present study, we measured 2D:4D of all four feet of adult male and female green anoles (*Anolis carolinensis* Voigt, 1832) to determine whether it is sexually dimorphic and whether results are repeatable across laboratories. Lizards were housed at Michigan State University (MSU) and Oklahoma State University (OSU), and one investigator at each institution used digital calipers to measure the 2D:4D of each foot. At both MSU and OSU, we found that males had a significantly larger 2D:4D on the back right foot than females did, and that no sex difference existed in either the back left or the front right foot. Furthermore, although no sex difference in the front left foot was found at MSU, the 2D:4D on this foot was larger in females at OSU. Our results demonstrate both sexual dimorphism in 2D:4D and repeatability between laboratories, but they also suggest the importance of verifying such repeatability if 2D:4D or any other digit length ratio is used as a potential indicator of the early steroid environment.

Résumé : Les rapports entre les longueurs des doigts sont déterminés au cours du développement embryonnaire et une exposition aux stéroïdes peut provoquer un dimorphisme sexuel. En particulier, le rapport du second sur le quatrième doigt (2D:4D) a été particulièrement étudié. Nous avons, dans notre travail, mesuré le rapport 2D:4D sur les quatre pieds des adultes mâles et femelles de l'anoles vert (*Anolis carolinensis* Voigt, 1832) pour voir s'il présente un dimorphisme sexuel et si les résultats peuvent être reproduits dans plusieurs laboratoires. Les lézards ont été gardés à Michigan State University (MSU) et à Oklahoma State University (OSU); un chercheur de chaque université a mesuré le rapport 2D:4D sur chaque pied à l'aide d'un pied à coulisse digital. Tant à MSU qu'à OSU, les mâles possèdent un rapport 2D:4D significativement plus grand au pied arrière droit que les femelles; il n'y a pas, cependant, de différence sexuelle sur le pied arrière gauche, ni sur le pied avant droit. De plus, alors qu'il n'existe pas de différence sexuelle au pied gauche avant à MSU, le rapport 2D:4D du même pied est plus grand chez les femelles à OSU. Nos résultats montrent qu'il y a un dimorphisme sexuel dans le rapport 2D:4D et que les résultats peuvent être reproduits d'un laboratoire à l'autre; cependant, ils indiquent qu'il est important de vérifier cette reproductibilité, lorsqu'on utilise 2D:4D ou tout autre rapport de longueur des doigts comme indicateur potentiel de l'environnement stéroïde au début de la vie.

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Introduction

Researchers have become increasingly interested in the existence of sexual dimorphism in digit length ratios, with the second-to-fourth digit length ratio (2D:4D) receiving

most of the attention. As was first demonstrated in humans, males have a smaller 2D:4D than females, on average (Phelps 1952), and this sex difference is more pronounced on the right hand (Manning et al. 1998; McFadden and Shubel 2002). Significant relationships between digit length ratio and a variety of sex-related traits, including a negative correlation with adult male testosterone concentration and sperm number, also have been identified (Manning et al. 1998). Such relationships indicate associated early developmental effects on both sex-related phenotypes and digit length ratios because these ratios are organized early in development and they demonstrate little to no subsequent plasticity (e.g., Garn et al. 1975; Brown et al. 2002a; Trivers et al. 2006). Thus, digit length ratios may provide stable adult indicators of an individual's embryonic environment.

In vertebrates, both differentiation of the urogenital system and growth and patterning of digits are regulated by homeobox genes, specifically *HoxA* and *HoxD* (Kondo et al. 1997; Peichel et al. 1997). One proposed mechanism for the coupling of digit length ratio with sexual differentiation is

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the influence of prenatal androgens on *HoxA* and *HoxD* expression within an individual (Brown et al. 2002a; Manning 2002). Brown et al. (2002a) gave support to this prenatal androgen hypothesis when they measured the 2D:4D of human females with congenital adrenal hyperplasia (CAH), a genetic condition leading to unusually high production of prenatal androgens. Females with and without CAH are genetically of the same sex, but females with CAH have male-like androgen levels during development. Measurements uncovered a smaller, “masculinized” digit ratio in females with CAH as compared with those without CAH.

Sexual dimorphism in 2D:4D and other digit length ratios may be taxonomically widespread given the conservative nature of *Hox* genes, and research thus far supports this claim, but with some interesting differences. Rodents exhibit a pattern of sexual dimorphism in 2D:4D similar to that in humans, with females having a larger ratio than males and with this sex difference being present only on the right, or more strongly on the right than on the left, in mice (Brown et al. 2002b; Leoni et al. 2005) and rats (McMechan et al. 2004). However, a recent study of Guinea baboons (*Papio papio* Desmarest, 1820) found a pattern dissimilar to that in rodents and humans, in which the 2D:4D of the right hand is larger in males rather than in females, though a negative correlation between adult male testosterone level and 2D:4D is still present (Roney et al. 2004). Burley and Foster (2004) were the first to report avian digit length ratios, and they found that in contrast to the pattern in rodents and humans, male zebra finches (*Taeniopygia guttata* Vieillot, 1817) have a larger 2D:4D than females do, at least for the right foot (results for the left foot were not reported). In addition, since mother zebra finches allocate decreasing amounts of androgen across laying order (Gil et al. 1999), smaller 2D:4D was correlated with higher maternally derived androgen levels in eggs laid earliest in a clutch, suggesting that early androgens decreased 2D:4D. However, Forstmeier (2005) reported no sexual dimorphism in digit length ratio and no laying order effect in a larger captive population of zebra finches ($n = 500$). Instead, strong additive genetic variation was demonstrated by cross-fostering, the strength of which varied between generations. Further, Romano et al. (2005) found that unmanipulated pheasants do not demonstrate sexual dimorphism in 2D:4D, though females hatched from testosterone-injected eggs had an increased 2D:3D on the left foot. Finally, in the first reported study addressing reptilian digit length ratios, Rubolini et al. (2006) found that male common wall lizards (*Podarcis muralis* Laurenti, 1768) have a larger left front and a larger right front 2D:4D, and a larger left front 2D:3D, than females do (hind feet were not measured). In contrast, these authors found no sex difference in 2D:4D, and a larger left front 2D:3D in females, in tree skinks (*Mabuya planifrons* Peters, 1878).

In the present study, we investigated 2D:4D in adult male and female green anole lizards, *Anolis carolinensis* Voigt, 1832, to further explore sexual dimorphism of digit length ratio in a comparative context. As indicated above, existing reports of 2D:4D in non-human species suggest different conclusions regarding the extent of sexual dimorphism and the relationships of other traits with 2D:4D. Extant reptiles share a common ancestor with avian species and together di-

apsid birds and reptiles represent a phylogenetic sister group to the synapsid mammals (Kumar and Hedges 1998). Owing to the evolutionary ties of reptiles to both avian and mammalian species used in recent studies of digit length ratios, a broader understanding of length ratio patterns in reptiles will provide insight into the extent to which this pattern is shared or derived across taxa. *Anolis carolinensis* is an excellent model for this study because the pre-hatching hormone environment has been well investigated. Increased testosterone exposure of males during early development, likely of both maternal and embryonic origin (Lovern and Wade 2001, 2003a, 2003b), may lead to differentiation of masculine and feminine traits, including sexual dimorphisms in digit elongation.

We had two specific objectives in undertaking this study. First, we compared the 2D:4D of all four feet of male and female green anoles to distinguish among three possible hypotheses regarding patterns in digit length ratio: (1) the phylogenetic constraint hypothesis, which states that closely related taxa will have more similar digit length ratios than distantly related taxa; (2) the genetic sex hypothesis, which states that species that share a genetic sex-determining mechanism will have digit length ratios more similar to each other's than to those of species with a different mechanism; and (3) the sexual monomorphism hypothesis, which states that no sex differences in digit length ratios are present. If the phylogenetic constraint hypothesis applies, green anoles should express a 2D:4D similar to that of birds, in which females have a smaller 2D:4D than males. This outcome would suggest that phylogeny exerts a strong influence over digit length ratio, since birds and extant reptiles share a more recent common ancestor than either does with mammals (Kumar and Hedges 1998). If the genetic sex hypothesis applies, green anoles should possess a mammal-typical 2D:4D, which is smaller in males. This outcome would suggest that genetic sex exerts a stronger influence on digit ratio than phylogeny does, because in mammals and reptiles such as anoles, males are the heterogametic sex, whereas in birds females are the heterogametic sex (Pough et al. 2004). This hypothesis is supported by the results of the studies reviewed above: with the exception of Guinea baboons, 2D:4D is smaller in the heterogametic sex, whether it be male mammals or female birds. Lastly, if the sexual monomorphism hypothesis is supported, green anoles will lack sex differences in 2D:4D altogether. Our second objective in this study was to examine the reproducibility of 2D:4D measurements between laboratory populations of green anoles. As digit length ratio is becoming an increasingly popular proxy for the early steroid environment, such reproducibility will be necessary. This is especially true given that, at least in one species, recent evidence suggests a high degree of genotype–environment interaction in digit length ratio (zebra finches; Forstmeier 2005).

Materials and methods

Animals and housing

Adult male and female anoles (defined as individuals with snout–vent lengths ≥ 45 mm) were housed under similar conditions in two laboratory colonies, one at Michigan State University (MSU) and one at Oklahoma State University

(OSU). Lizards in the MSU colony were obtained from Charles Sullivan (Nashville, Tennessee, USA) and those in the OSU colony were purchased from Carolina Biological Supply Company (Burlington, North Carolina, USA). We know that all lizards in the study were wild-caught adults from a variety of locales in Louisiana and Florida, but otherwise precise ages were unknown, as were degrees of relatedness. We assume that individuals were at least 1 year old, based on growth rates and time to maturity for this species (reviewed in Lovern et al. 2004), and that relatedness was low within and nonexistent between laboratory colonies.

Lizards in both colonies were maintained under standard conditions (e.g., Lovern et al. 2004). They were either housed in groups of 1 male and 3–7 females in 110 L glass aquaria or housed individually in 38 L glass aquaria. Each aquarium contained a peat moss substrate and at least one rock and one dowel for perching and basking. Room conditions were set to mimic natural breeding conditions. A 14 h light : 10 h dark cycle was maintained with a combination of fluorescent, full-spectrum, and incandescent heat lamps. Temperatures in aquaria ranged from 25 to 38 °C during the day, depending on distance from the heat lamps, to 18 °C at night. Relative humidity was maintained at 60%–75%. Cages were misted daily with water as an additional source to that provided ad libitum in shallow water bowls, and lizards were fed mealworms or vitamin-dusted crickets at least three times each week.

Digit measurements

We attempted to standardize measurement of digit lengths as much as possible between the MSU and OSU laboratories. We used identical digital calipers (Plasti-cal digital calipers, Mitutoyo, Aurora, Illinois, USA) to measure the lengths of the second and fourth digits of each foot to the nearest 0.1 mm, with one individual performing all of the measurements at each laboratory (J.L.C. at MSU and S.D. at OSU). Other digits were not measured. Each lizard was restrained by hand to isolate the digits to be measured, and length was measured twice for each digit and recorded. Repeatability was high between pairs of measurements, as assessed by correlation analysis ($r = 0.97$ for MSU and 0.96 for OSU). Duplicate values were averaged for each digit and then the ratio of second to fourth digit length was calculated for each foot for all analyses. On each foot, the medial digit was considered the first digit and the lateral digit was considered the fifth. Measurements were made with the zero caliper tip placed on the basal crease of the digit and the moving tip placed distally at the point where the nail emerges. In total, we measured 45 females and 38 males at MSU and 42 females and 23 males at OSU. However, for animals that had been previously toe-clipped for identification, measurements were taken only from those feet that did not have a second or fourth digit clipped; thus, we did not obtain four ratios for all individuals.

Statistical analyses

The 2D:4D was not normally distributed for any of the four feet (Kolmogorov–Smirnov tests; all $p \leq 0.05$), and there was an inequality in variance between the sexes for the back left 2D:4D (Levene's test; $p = 0.001$). Because data transformations did not result in normality or homoge-

Table 1. Results of a general linear model ANOVA for effects of laboratory and sex on the second-to-fourth digit length ratio of green anoles (*Anolis carolinensis*).

Foot	Effect		
	df	<i>F</i>	<i>p</i>
Back right	1, 1, 126		
Lab		30.84	<0.001
Sex		14.49	<0.001
Lab × sex		0.02	0.899
Back left	1, 1, 129		
Lab		34.08	<0.001
Sex		3.42	0.067
Lab × sex		1.70	0.195
Front right	1, 1, 129		
Lab		12.56	0.001
Sex		0.97	0.326
Lab × sex		0.07	0.791
Front left	1, 1, 131		
Lab		32.76	<0.001
Sex		3.52	0.063
Lab × sex		9.37	0.003

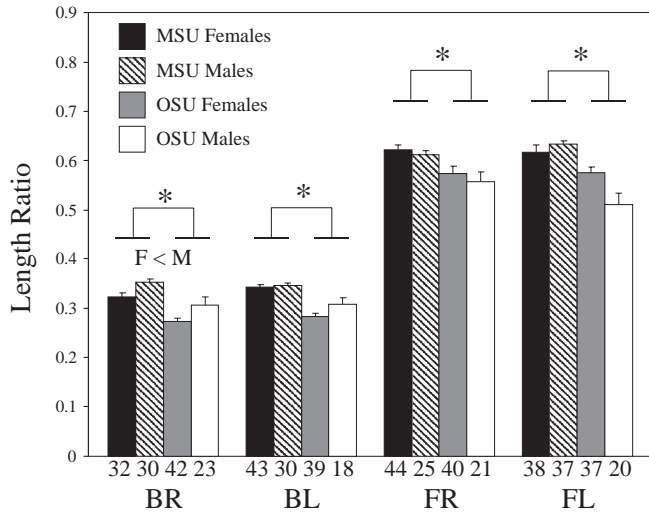
neity of variance, we used Kruskal–Wallis tests to examine the effects of laboratory (MSU vs. OSU) and sex (female vs. male) independently for each 2D:4D (i.e., back right foot (BR), back left foot (BL), front right foot (FR), and front left foot (FL)). We compared these results with those obtained with general linear model ANOVAs, and because there were no differences between the resulting interpretations (i.e., significant and nonsignificant results were identical between the tests), we report the results obtained from ANOVAs. To address the reproducibility of results between MSU and OSU, we compared the outcomes of Kruskal–Wallis tests calculated separately for each laboratory population. We also looked for a possible sex difference in asymmetry between the left and right digit length ratios, for the front and back feet separately, by subtracting the right ratio from the left ratio and analyzing the resulting values with Kruskal–Wallis tests.

Results

Effects on 2D:4D due to lab, sex, or their interaction were observed, depending upon the ratio in question (Table 1). Only BR 2D:4D showed a significant sex difference ($F = 14.49$, $p < 0.001$); for females the ratio was 0.294 ± 0.006 and for males it was 0.333 ± 0.007 (Fig. 1). In contrast, all four digit length ratios showed significant laboratory effects (all $p \leq 0.001$) (Table 1). There was a sex × laboratory interaction on FL 2D:4D ($F = 9.37$, $p = 0.003$), which was larger in females than in males at OSU but smaller in females than in males at MSU.

Reproducibility of results between MSU and OSU was high (Fig. 1). That is, female BR 2D:4D was significantly smaller than male BR 2D:4D for both laboratory populations, and there were no significant differences between sexes for BL or FR (Table 2). Consistent with the sex × laboratory interaction reported above, FL 2D:4D of females was significantly larger than that of males for the OSU pop-

Fig. 1. Comparisons of second-to-fourth digit length ratios across laboratory and sex for green anoles (*Anolis carolinensis*). BR, back right foot; BL, back left foot; FR, front right foot; FL, front left foot. Sample sizes are given below the bars. Asterisks above bars indicate significant effects due to laboratory ($p \leq 0.001$). F < M indicates that females have a significantly smaller ratio than males ($p < 0.001$). See Table 1 for complete results of statistical analyses.



ulation, but no such sex difference was present in the MSU population (Table 2). Finally, no sex differences in asymmetries were detected between the left and right digit length ratios for MSU and OSU data, analyzed separately or combined (Kruskal–Wallis tests; all $p > 0.05$). The asymmetry of the back feet was marginally significantly different, however, in the combined data set for females (0.012 ± 0.007) versus males (-0.005 ± 0.006) ($H = 2.91$, $p = 0.088$).

Discussion

In the present study we document that male green anoles have a significantly larger BR 2D:4D than females do and that this result is repeatable across laboratories. We also document consistent laboratory effects on 2D:4D measurements, but only in one case did these effects lead to different conclusions. OSU females had a significantly larger FL 2D:4D than OSU males did, but MSU females and males exhibited no sexual dimorphism in this particular digit length ratio.

Our results for 2D:4D are most consistent with the phylogenetic constraint hypothesis proposed in the Introduction. Similar to zebra finches (Burley and Foster 2004; but see Forstmeier 2005) and wall lizards (Rubolini et al. 2006), green anoles possess a male-biased dimorphism in 2D:4D, in contrast to the female-biased dimorphism seen in mammals (other than Guinea baboons; Roney et al. 2004). Collectively, these results suggest that when sexual dimorphism in 2D:4D is present, reptiles and birds show more similarities to each other than either taxa does to mammals, following evolutionary relatedness rather than a genetic sex-determining mechanism. Thus, although the available data are limited at this point, it may be that a common ancestor of both birds and lizards evolved this variation in the digit length ratio pattern and that the variation has been phylogenetically conserved among extant diapsids.

Table 2. Results from Kruskal–Wallis tests comparing the second-to-fourth digit length ratio between male and female green anoles separately for MSU and OSU populations.

Foot	MSU		OSU	
	<i>H</i>	<i>p</i>	<i>H</i>	<i>p</i>
Back right	6.94	0.008	4.18	0.041
Back left	0.01	0.964	3.10	0.078
Front right	0.50	0.481	0.11	0.738
Front left	0.01	0.958	8.66	0.003

Measurements of digit length ratios in more reptilian and avian species would allow for a more thorough examination of this hypothesis.

The sexual dimorphism in 2D:4D for the back right foot demonstrated in the present study of green anoles is consistent with what generally is seen in rodents (e.g., Brown et al. 2002b; Leoni et al. 2005). Additionally, evidence from zebra finches (Burley and Foster 2004) and wall lizards (but not tree skinks; Rubolini et al. 2006) supports the tentative conclusion that across taxa, right limbs exhibit a stronger sex difference in digit length ratios than left limbs do. However, it is curious that dimorphisms tend to be stronger in the forelimbs of primates and the hind limbs of four-legged non-primates. Determination of whether this trend indicates a phylogenetic divergence in primates or is just a result of experimental bias or measurement error (e.g., toes can be more difficult to measure than fingers; McFadden and Shubel 2002) will require more reports of both front and back limb measurements across species.

Our results are also consistent with the hypothesis that sexual dimorphism in 2D:4D is produced by sex differences in androgens during early development, likely via a simultaneous influence on *HoxA* and *HoxD* expression for the digits and genitals (e.g., Manning 2002). A timeline of these events can be constructed using the timing of genital development across species along with what is known about green anole testosterone exposure and the phalangeal development documented for two additional lizard species (Fig. 2). This timeline suggests two specific hypotheses for green anoles. First, hemipenes (bilateral adult male lizard genitalia, which develop in male embryos and regress in female embryos) differentiate and digits elongate together during embryonic days 11–18 (hatching occurs around day 35). Second, at least some of the testosterone that influences these concurrent events is likely of maternal origin, via testosterone deposition into yolk. This hypothesis is supported by the fact that yolk testosterone content is high during early development, whereas testosterone production by embryos appears to be minimal until sometime closer to day 24 (Lovern and Wade 2003a). Support for the hypothesis that the androgens affecting digit length ratios are maternally derived is also available from the study of hormone allocation in yolk and effects on 2D:4D in zebra finches by Burley and Foster (2004). Future studies investigating the influence of exogenous hormones applied at 11–18 days in comparison with other time points might be useful to determine whether digit ratio patterns are a time-sensitive indicator of the hormone environment during development.

Fig. 2. Timeline of lizard genital and phalangeal development and testosterone (T) exposure. Oviposition is at day 0 (stage 29), and hatching is at days 34–35 (stages 41–42). This timeline outlines peaks in green anole T levels for both maternally derived yolk T and offspring-derived embryo T as reported in Lovern and Wade (2003a). Also, as described in Holmes and Wade (2005), events relating to sexual differentiation in *Anolis carolinensis* include the existence or regression of bilateral hemipenes, the paired adult male copulatory organs that first appear in both sexes but regress in females. Finally, the hemipene stage of *A. carolinensis* is compared with the developmental stages of Dufaure and Hubert (1961) for the common lizard (*Lacerta vivipara* Jacquin, 1787) and Muthukkaruppan et al. (1970) for the garden lizard (*Calotes versicolor* Daudin, 1802) to extrapolate digit elongation to the timeline. The double asterisk (**) indicates days during *A. carolinensis* embryonic development that are particularly important for understanding 2D:4D (see Discussion).

Event:	Oviposition	First yolk T peak	♂ & ♀ hemipenes	Digits elongate; ♀ hemipenes regress	♀ hemipenes regressed	Second yolk & first embryo T peak	Second embryo T peak	Hatching
Anolis Day	0	8	9-10	11-18**	19	24	32	34-35
Lacerta stage	29	32	33	34	36	38-39	41	41-42
Calotes stage	29	32	33	34	38	39-40	41-42	42

Measurement of digit length ratios such as 2D:4D may be a valuable tool as an indicator of early hormone exposure, particularly androgens, due to the likelihood that digit length ratio is organized by androgens during embryonic development but affected little, if at all, by subsequent hormone exposure (Garn et al. 1975; Breedlove et al. 1998; Trivers et al. 2006). This morphological stability after birth may allow for a relatively simple evaluation of differences in androgen exposure experienced during development for groups of individuals within a species. However, in order for digit length ratio to be a reliable tool for a given species, the reproducibility of sex differences should be assessed, not only across populations but also across generations within the same population (Forstmeier 2005). The disparity between MSU and OSU FL 2D:4D results, for example, could indicate a difference between the wild populations from which these animals were sampled or that methods of measurement used by MSU and OSU experimenters were not identical despite a concerted effort to standardize these procedures. Our results show that for green anoles there is a reproducible sex difference in BR 2D:4D across populations. Whether sex differences exist in other digit length ratios, whether the sex difference in BR 2D:4D is stable across generations, and whether such differences directly relate to embryonic androgen exposure remain to be experimentally determined.

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