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Hemipenial Allometry in *Anolis grahami*

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ABSTRACT.—In animals with internal fertilization, the male genitalia often vary greatly, have complex morphology, and show fast rates of evolution. In studies of the evolution of such male genitalia, static allometric relationships have been used extensively. Static allometry is an intraspecific measure of proportional size of a particular structure with respect to the body size. We examined the static allometric pattern of the genitalia of two allopatric subspecies of a Jamaican *Anolis* lizard, *Anolis grahami grahami* and *A. grahami aquarum*, and compared our findings to observed patterns in nongenital traits: limbs and the dewlap. Limb and dewlap traits showed similar allometric slopes, with some variation in the intercept, whereas hemipenial traits showed different slopes. The hemipenial traits of *Anolis g. grahami* exhibited a statistically significant negative allometry, while *A. g. aquarum* showed slope values that were not statistically different from isometry. Our results corroborate the idea that genital traits evolve faster than other morphological body traits.

For several decades there has been increasing interest in understanding the evolution of male genital traits (Eberhard, 1985, 2010; Arnqvist and Thornhill, 1998; Rowe and Arnqvist, 2011). In addition to the direct relationship to reproduction and fitness within a population, male genitalia are important in the speciation process (Coyne and Orr, 2004). Between closely related species, male genitalia often show astounding variation in size, shape, and elaboration and are often more divergent than nongenital traits (Arnqvist, 1997; Simmons and Jones, 2007; Eberhard, 2010).

The study of static allometric relationships has been used extensively to describe morphological variation in genitalia across species (Eberhard, 2009) but its utility to explain evolutionary patterns can be limited (Bonduriansky, 2007). Static allometry is an intraspecific measure of proportional size of a particular structure with respect to body size, often calculated as the slope from a regression of the trait of interest against body size across multiple individuals in a population (Eberhard et al., 1998). Variation in allometric slopes among different classes of traits (e.g., genital vs. nongenital) within a population may suggest hypotheses about the nature of selection acting on those traits and the rate at which they are evolving (Bernstein and Bernstein 2002; Eberhard, 2009). Bonduriansky (2007) discussed how positive static allometry patterns have been associated with sexual traits in certain groups; however, those conclusions were drawn exclusively from species with unusually exaggerated traits (e.g., beetle horns and Irish Elk *Megaloceros giganteus* antlers). Bonduriansky (2007) showed that traits under sexual selection do not necessarily lead to the evolution of positive static allometry, however, and positive static allometry is not necessarily a consequence of sexual selection. Eberhard (2009) reviewed allometric patterns for male genitalia across 135 animal species with internal fertilization; 75% of the available data are from arthropods and, in most arthropod species, male genitalia have negative allometric slopes that are comparatively more negative than nongenital traits. These results were interpreted as evidence of stabilizing sexual selection favoring male genitalia

that matched the average female genital size in the population (Eberhard 1985; Eberhard, 2009).

Despite such extensive work on the allometry of male genitalia in insects and spiders (Eberhard, 2009), few allometric data exist for vertebrates and no quantitative data have been taken for squamate reptiles. At best, the available data consist of qualitative descriptions (Eberhard, 2009).

The genitalia of male squamates consist of a pair of intromittent organs called hemipenes. The hemipenes are tubular structures that are retracted into the base of the tail when not in use but are everted during copulation. Each hemipenis surface contains a groove, the sulcus spermaticus, through which semen is conducted. Hemipenial morphology varies extensively among squamate taxa, from cylindrical tubes to deeply bilobed structures ornamented with calyces, papillae, flounces, and spines (Dowling and Savage, 1960) and thus has been used extensively for systematic studies in snakes (Dowling and Duellman, 1978; Zaher, 1999; Myers and Donnelly, 2001; Schargel and Castoe, 2003; Schargel et al., 2005) and, to a lesser extent, in lizards (Arnold, 1983, 1986; Köhler and Hahn, 2012; Nunes et al., 2012).

We examined the static allometric pattern of the hemipenis of two Jamaican sister lineages: *Anolis grahami grahami* and *A. grahami aquarum*. We obtained three measurements of the hemipenis: hemipenial length, width at the lobe, and width at the hemipenial body. We also measured the length of the thigh, shank, and the length of the second arc ceratobranchial of the hyoid as a proxy for dewlap size. The leg traits are likely under natural selection (Losos, 2009) while the dewlap is used primarily for social signaling and is considered essential to species recognition (Glor and Laport, 2012; Harrison and Poe, 2012). Thus, the dewlap serves as a nonhemipenial trait that may yet be under sexual selection and is useful for comparison. We used snout–vent length (SVL) as a measure for body size. We questioned and addressed three aspects of hemipenial evolution. First, we examined the static allometry of hemipenial variation (Question 1). Second, we compared the allometric slopes of hemipenial and nonhemipenial traits (Question 2). Finally, we examined whether differences exist between the two lineages (Question 3).

MATERIAL AND METHODS

Study Organism.—*Anolis grahami* is a Jamaican species most often found on trees from eye level to high in the canopy (a

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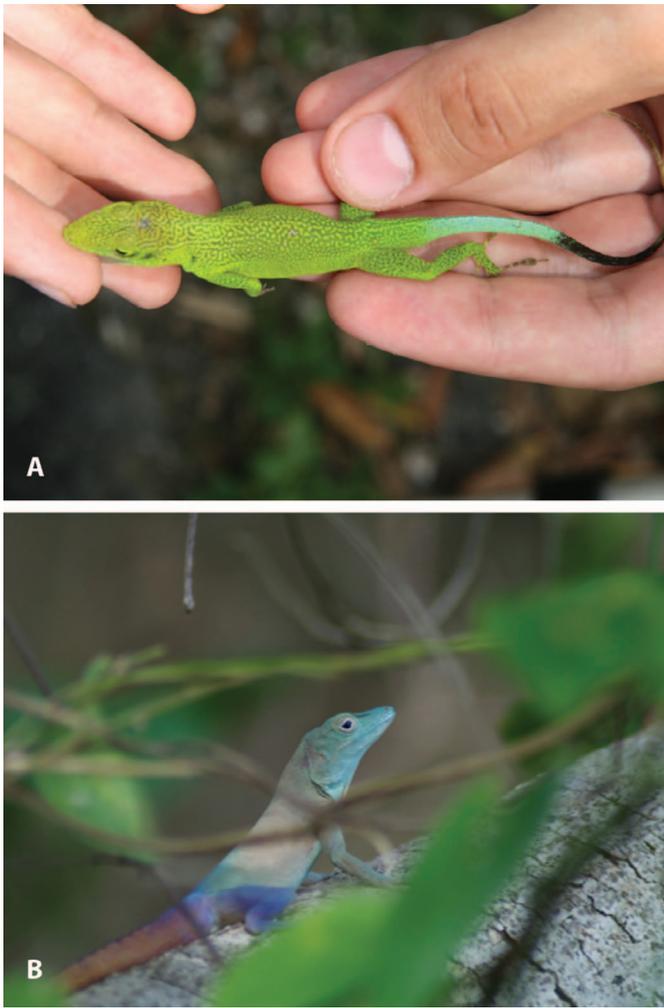


FIG. 1. (A) *Anolis grahami aquarum* and (B) *A. g. grahami*. Photo credit: Brent Berger.

member of the “trunk-crown” ecomorph, sensu Williams, 1983). There are two recognized subspecies: *A. g. grahami* and *A. g. aquarum*, which differ consistently in coloration and squamation (Fig. 1). *Anolis grahami aquarum* is restricted to the east side of Jamaica and is a bright, emerald green, whereas *A. grahami grahami* is distributed in the central and western parts of the island and often displays motley colors on a single individual including orange, blue, yellow, purple, and green (Fig. 1). On the south coast of the island, the boundary between the two subspecies is well defined by the Morant River; however, on the north coast a contact zone exists across a several-kilometer stretch and intermediate forms have been described (Underwood and Williams, 1959; YS, pers. obs.). The subspecies do not inhabit the mountains between the two coastal contact zones. We analyzed 29 male specimens of *A. g. grahami* and 13 specimens of *A. g. aquarum*, sampled on either side of the subspecies boundary (Appendix 1). All the specimens were collected by hand or with noose-poles between July and August of 2008 during the species reproductive season (Licht and Gorman, 1970). In the field, animals were euthanized with a sodium pentobarbital injection and the hemipenes were everted manually, partially or completely, before the specimen was fixed in formalin 10%. The specimens were deposited in the Herpetological Collection of the Museum of Comparative Zoology (MCZ) of

Harvard University, Cambridge, Massachusetts, USA (Appendix 1).

Hemipenial Preparations.—One of the hemipenes was dissected from each fixed specimen through a small incision at the base of the tail. The removed organ was immersed in a 2% KOH solution for 3–5 min or until it became translucent and flexible. The hemipenis was checked and everted manually once more using forceps to be sure that all specimens were completely everted. Once fully everted, the organ was filled with colored Vaseline to allow better visualization of ornamentation structures. The hemipenes were prepared following Zaher and Prudente (2003) for snake organs. We obtained digital images of hemipenes in sulcate view using a JVC camera, KYF75U, attached to a stereomicroscope with the aid of Auto-Montage Pro, 5.02.

Morphological Measurements.—We used SVL as a proxy for body size. We used a digital caliper to measure length of the thigh, length of the shank, length of the dewlap, hemipenial total length, hemipenial width at the lobes, and hemipenial width at the hemipenial body on each male. Thigh length was measured ventrally from the insertion point of the head of the thigh to the knee while shank length was measured dorsally. Dewlap length was measured as the length of the second arc ceratobranchial of the hyoid from lizard snout to the end of the cartilage; we had to expose the final portion of the second arc ceratobranchial cartilage through a small incision at the base of the dewlap. We measured the hemipenial dimensions from digitized images using the software ImageJ 1.46r (Rasband, 1997–2012) as follows. We traced an imaginary line across the apex of the lobes and used the measure of the distance between the imaginary line and the base of the hemipenis as the hemipenial length. The width at the hemipenial lobes was obtained by measuring the wider region of the lobes, whereas width at the hemipenial body was obtained by measuring the central area of the body of the hemipenis (Fig. 2).

All measurements were repeated twice. We calculated the repeatability of the measurements using the intraclass coefficient (ICC) as described by (Lessells and Boag, 1987) using the ICC package (Wolak, 2012) in R software (R Core Team, 2013). For the subsequent statistical analysis we used the mean of both measurements, and all the measurements were log transformed.

Statistical Analysis.—We tested whether the measured traits showed allometric relationships with body size (Question 1) by estimating the coefficient of allometry (b) as the slope of a linear regression of trait values against a measure of body size (Eberhard et al., 1998), in this case, SVL. For each trait we tested whether the slopes of the regression were significantly different from the null hypothesis of isometry ($b_{OLS} = 1$) using a t -test. Slope values higher than one show positive allometry while slope values smaller than one show negative allometry.

To ask whether allometric relationships differ between hemipenial and nonhemipenial traits (Question 2), we quantified phenotypic variation using the coefficient of variation (CV); that is, the dispersion around the mean calculated as the standard deviation of each trait divided by its mean (Eberhard et al., 1998; Bernstein and Bernstein, 2002). We also estimated the dispersion around the reduced major axis (CV'); that is, the coefficient of variation that the trait would have if body size were held constant (Bertin and Fairbairn, 2007; Eberhard et al., 2009).

The allometric slopes and intercepts for each subspecies were compared (Question 3) using an analysis of covariance (ANCOVA) (Sokal and Rohlf, 1995). All analyses were



FIG. 2. Hemipenis in sulcate view of *A. g. grahami* (MCZR-192575): (1) measure of hemipenial total length; (2) measure of hemipenial width at the lobes; and (3) measure of hemipenial width at the body.

performed using JMP (JMP®, Version 11, SAS Institute Inc., Cary, NC, 1989–2007).

RESULTS

The mean, standard error, and the ICC coefficient of the hemipenial and nongenital measurements for both taxa are summarized in Table 1. Allometric slope for dewlap length was not significantly different from 1.0, but slopes for the limbs measurements were all significantly less than 1.0 (Table 2). Allometric slopes for the three hemipenial measurements of *A. grahami grahami* were also significantly less than one (all $P \leq 0.007$) (Question 1). Furthermore, when compared to both the dewlap and limb traits, *A. g. grahami*'s hemipenial slopes were significantly smaller (Table 2) (Question 2).

In contrast, allometric slopes for *A. g. aquarum* were not significantly different from 1.0 for any of the traits measured (hemipenial and nonhemipenial; Question 1) and, unlike for *A. g. grahami*, *A. g. aquarum* hemipenial traits had larger slopes than did nonhemipenial traits, although the differences were not significant (Question 2).

TABLE 1. Descriptive statistics for hemipenial and nongenital traits: mean, standard error (SE), and the intraclass coefficient (ICC) showing repeatability.

	Sample size	Mean (mm)	SE	ICC
<i>Anolis g. aquarum</i>				
	13			
Dewlap		32.25	0.87	0.98
Thigh		12.60	0.27	0.95
Shank		7.91	0.16	0.89
Hemipenis length		6.33	0.22	0.99
Hemipenis width at lobe		4.58	0.21	0.99
Hemipenis width at body		2.24	0.09	0.98
<i>Anolis g. grahami</i>				
	29			
Dewlap		34.25	0.79	0.98
Thigh		12.45	0.19	0.93
Shank		7.85	0.11	0.91
Hemipenis length		6.17	0.10	0.91
Hemipenis width at lobe		4.28	0.08	0.99
Hemipenis width at body		2.21	0.04	0.98

The measures of phenotypic variation showed a similar pattern to allometric slopes in both subspecies: hemipenis traits had higher CV values than body traits (Table 1). The CVs for the hemipenial traits varied between 4.9–16.7%, whereas the body traits exhibited values between 2.6–3.6%. The dispersion around the major axis (CV') showed smaller overall values, but the same pattern was observed with hemipenial traits showing slightly higher values than did nonhemipenial traits (Table 2) (Question 2).

The only significant difference in allometry between subspecies (Question 3) was in the dewlap intercept (Table 3). The slopes of the hemipenial traits did not differ significantly between the subspecies for each trait, although the hemipenial slopes for *A. g. aquarum* were larger than those obtained from *A. g. grahami* (Table 2). Limb and dewlap allometric slopes are similar across subspecies (Table 2).

TABLE 2. Summary statistics for ordinary least square (OLS) regression of trait vs. body size (SVL). Allometric coefficient b , the slopes of OLS; coefficient of determination r^2 ; t -value; significance of deviation from a slope of one (P); CV, coefficient of variation; and CV' dispersion around the reduced major axis.

	b	r^2	t -value	P	CV	CV'
<i>Anolis g. aquarum</i>						
Dewlap	0.986	0.699	-0.063	0.95	2.618	2.959
Thigh	0.781	0.714	-1.327	0.21	2.814	2.539
Shank	0.740	0.652	-1.443	0.18	3.418	2.818
Hemipenis length	0.782	0.281	-0.523	0.61	6.177	5.779
Hemipenis width at lobe	1.277	0.431	0.567	0.58	9.926	6.069
Hemipenis width at body	1.216	0.493	0.526	0.61	16.68	5.161
<i>Anolis g. grahami</i>						
Dewlap	1.088	0.689	0.614	0.54	3.483	3.899
Thigh	0.822	0.803	-2.277	0.03	3.384	2.493
Shank	0.721	0.795	-3.960	0.001	3.65	2.419
Hemipenis length	0.451	0.221	-3.367	0.002	4.913	5.762
Hemipenis width at lobe	0.441	0.176	-3.045	0.005	6.743	6.312
Hemipenis width at body	0.433	0.154	-2.898	0.007	12.99	6.632

TABLE 3. ANCOVA results for differences between species, F -statistics, and P -values for each analyzed trait.

Trait	Slope		Intercept	
	F	P	F	P
Dewlap	0.1132	0.7386	21.7548	<0.0001
Thigh	0.0521	0.8208	3.3151	0.077
Shank	0.0126	0.9113	4.1539	0.0489
Hemipenis length	0.7051	0.4066	0.0148	0.904
Hemipenis width at lobe	3.4497	0.0715	0.8441	0.3643
Hemipenis width at body	3.0939	0.0871	0.2148	0.6458

DISCUSSION

The extreme variation in male genitalia morphology is a remarkable phenomenon that has interested biologists for decades (Eberhard, 2010). The study of genital variation should help not only to clarify the processes driving morphological evolution but also to understand the mechanisms of reproductive isolation and speciation (Oneal and Knowles, 2012). Indeed, closely related species often show diverged genitalia without remarkable differences in the nongenital morphology (Arnqvist, 1997), suggesting that genital traits may evolve rapidly and be an important component of reproductive isolation (Eberhard, 2009).

Consistent with this idea, our results show that *Anolis grahami* subspecies have hemipenial traits that differ in allometric pattern: *Anolis g. grahami*'s hemipenial traits exhibited a statistically significant negative allometry while *A. g. aquarium* showed slope values that were not statistically different from isometry (Fig. 2, Table 1). Moreover, while *A. g. grahami* and *A. g. aquarium* differ in allometric patterns for hemipenial traits, they do not differ in allometric patterns for nonhemipenial traits (Fig. 2, Table 2), altogether suggesting that hemipenial evolution

is under selection and could play a role in the speciation process in this incipient species pair.

Sexual selection on male hemipenial traits, particularly cryptic female choice, may explain our results. Under cryptic female choice, male genitalia have a stimulatory function and females choose to use sperm from males with superior stimulatory capabilities. A corollary of this hypothesis is the idea of the "one-size-fits-all" strategy: regardless of body size, males would be selected to have genitals that fit the average size of female genitals in the population (Eberhard, 1985). If "one-size-fits-all" were at play, then negative allometry and relatively smaller slopes compared to other morphological traits would be predicted as a result of stabilizing sexual selection, as seen in studies of genital allometry in insects and arthropods (Eberhard et al., 1998, 2009). Our finding of negative allometry and smaller relative slopes in *A. g. grahami* suggests that cryptic female choice may be important in this system. However, we cannot rule out the possibility of stabilizing selection for lock-and-key hypothesis. The lock-and-key hypothesis suggests that selection for pre-insemination reproductive isolation will shape a species-specific male genitalia that fit appropriately in female genitalia (Anqvist, 1997).

Our results contrast with the few available data from other vertebrates, which generally show an opposite pattern of positive allometric slopes in male genitalia (Eberhard, 2009; Retief et al., 2013). This pattern has been interpreted as a result of directional sexual selection, where females choose males based on the relative size of genital traits, perhaps using genital size as an indicator of good genes (Pomiankowski and Møller, 1995).

High phenotypic variance, a possible indicator of opportunity for selection (Pomiankowski and Møller, 1995), has been associated with sexual selection. Our results show relatively higher values of CV and CV' for the hemipenial traits compared to other traits, suggesting that sexual selection may be driving

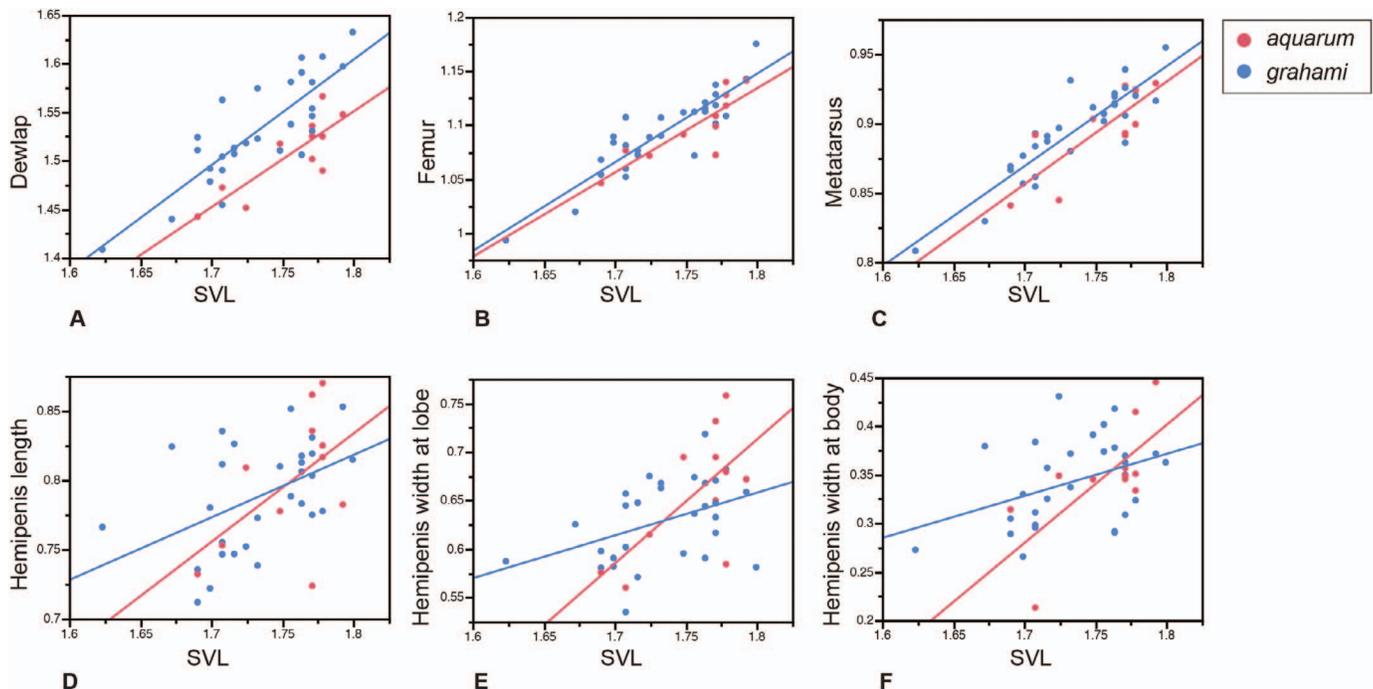


FIG. 3. Regressions of log-transformed traits vs. log-transformed body size (SVL): (A) dewlap; (B) thigh; (C) shank; (D) hemipenis total length; (E) hemipenis width at the lobes; and (F) hemipenis width at the body.

differentiation in genital morphology. These values are below the average CV described for traits under sexual selection (22.3%) but within the range of values for genital traits (3.3–90.5%) that are thought to be under sexual selection (Pomiankowski and Møller 1995; Eberhard, 2009).

Our work is among the first to describe allometric patterns of the squamate hemipenis. We show intraspecific variation in the hemipenis, with two closely related subspecies showing different hemipenial allometric patterns. While we are unable to determine the processes underlying the observed divergence in hemipenes, our work represents a necessary first step to clarify the processes responsible for hemipenial evolution.

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Anolis grahami aquarum: R-192574, R-192576, R-192581, R-192584, R-192606, R-192607, R-192608, R-192609, R-192610, R-192611, R-192613.

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A. grahami grahami: R-192575, R-192577, R-192578, R-192579, R-192580, R-192582, R-192583, R-192585, R-192586, R-192587, R-192588, R-192589, R-192590, R-192591, R-192592, R-192593, R-192594, R-192595, R-192596, R-192597, R-192598, R-192599, R-192600, R-192601, R-192602, R-192603, R-192604, R-192605, R-192612.

APPENDIX 1

List of Analyzed Material.—All the specimens are accessioned in the Museum of Comparative Zoology (MCZ), Harvard University, Cambridge, Massachusetts, USA.