**Ontogenetic allometry constrains cranial shape of the head-first burrowing worm
lizard *Cynisca leucura* (Squamata: Amphisbaenidae)**

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Short title: *Cynisca leucura* cranial ontogeny

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Abstract: Amphisbaenians are fossorial, predominantly limbless squamate reptiles with distinct cranial shapes corresponding to specific burrowing behaviors. Due to their cryptic lifestyles and the scarcity of museum specimens, little is known of their intraspecific variation, particularly regarding cranial osteology. This represents a critical lack of information, since the majority of morphological investigations of squamate relationships are based on cranial characters. We investigated cranial variation in the West African Coast Worm Lizard *Cynisca leucura*, a round-headed member of the family Amphisbaenidae. Using geometric morphometric analyses of three-dimensional computed tomographic scans, we found that cranial osteology of *C. leucura* is highly conserved, with the majority of shape changes occurring during growth as the cranium becomes more slender and elongate, accompanied by increasing interdigitation among the dermal roofing bones. Elements of the ventral portion of the cranium remain loosely connected in adults, possibly as a protective mechanism against repeated compression and torsion during burrow excavation. Intraspecific variation was strongly correlated with size change from juveniles to adults, indicating a dominant role of ontogenetic allometry in determining cranial shape. We found no evidence of sexual dimorphism, either during growth or among adults. Given the fossorial habits of *C. leucura*, we hypothesize that cranial allometry is under strong stabilizing selection to maintain adequate proportions for head-first digging, thereby constraining the ability of individuals to respond to differing selection pressures, including sexual selection and variation in diet or microhabitat. For species in which digging imposes less mechanical stress (e.g., in softer sand), allometric associations during growth may be weakened, allowing changes to the ontogenetic trajectory and subsequent morphological traits. Such developmental dissociation between size and shape, known as heterochrony,

may also be implicit in the evolution of the other amphisbaenian cranial shapes (shovel, spade and keel), which may themselves be functionally [adapted](#) for their respective burrowing techniques.

Key words: Amphisbaenia, *Cynisca leucura*, ontogeny, allometry, cranial shape

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Introduction

Amphisbaenians, or worm lizards, comprise nearly 200 species of small fossorial reptiles inhabiting tropical and semitropical regions of the world, of which all but three are limbless (Kearney, 2003a). Since separating from their closest relatives, lacertid lizards (Townsend et al., 2004; Vidal et al., 2008; Müller et al., 2011; Wiens et al., 2012), amphisbaenians evolved numerous adaptations for underground locomotion, including four distinct cranial shapes (shovel, spade, keel, and round) each associated with a particular head-first burrowing technique (Gans, 1974). Although some studies place two of the cranial morphotypes in monophyletic groups (shovel and keel-headed; Vanzolini, 1951; Kearney, 2003a), the high degree of conflict between morphological and molecular data has made phylogenetic reconstructions problematic. For example, morphological analyses recover the round-headed *Bipes* (the only genus with (fore)limbs) as most primitive (Gans, 1978; Kearney, 2003a), while molecular evidence places the shovel-headed Rhineuridae at the base of the tree with *Bipes* nested deep within the other limbless taxa (Kearney and Stuart, 2004; Macey et al., 2004; Vidal et al., 2008; Mott and Vieites, 2009). Which of these hypotheses, if any, is correct has significant consequences on our interpretations of character homology and limb loss (or gain).

Regardless of exact relationships, the Amphisbaenidae represents the most diverse (>170 species) and widespread amphisbaenian family, distributed across the Caribbean, sub-Saharan Africa, and South America (Gans, 2005). It is also the most disparate, with extant forms encompassing three of the four cranial morphologies (Kearney, 2003a). The

fourth cranial shape (spade-headed) is found in the Miocene African amphisbaenid *Listromycter leakeyi* (Charig and Gans, 1990).

Despite their relatively high diversity, few attempts have been made to characterize variation in amphisbaenid species in detail, particularly in terms of morphology but also regarding life history, behavior, diet, and geographical range (but see Montero et al., 1999; Vega, 2001; Ballestrin and Capellari, 2001; Vanzolini, 2002). In fact, many species are only known from the type or type series (see Gans, 2005), making estimates of intraspecific variation difficult. This is particularly true for features of the skull, which are often the focus of systematic investigations because they show marked interfamilial and intergeneric differences (Gans, 1978). Considering the high degree of variation reported for some conspecifics even from the same locality (e.g., one or two head shields among individuals of *Monopeltis guentheri*; Loveridge, 1941), understanding the range of morphological variation in amphisbaenid (and amphisbaenian) species would greatly expand our knowledge of this enigmatic group, and likely contribute to better supported phylogenetic hypotheses.

In this study, we examine intraspecific variation in the West African Coast Worm Lizard *Cynisca leucura*, a relatively small (<250 mm snout-vent length) round-headed member of the Amphisbaenidae. We focus on variation in cranial osteology using high-resolution X-ray computed tomography (CT), a non-destructive technique for visualizing internal anatomy of whole specimens. CT is particularly suitable for investigations of the amphisbaenian skull, since: 1) they are typically small (the skull of this species measures <1 cm in length); 2) they comprise overlapping and tightly compressed bones that are difficult to examine through traditional preparation; and 3) it circumvents the need to

disarticulate (but not destroy; see Bell and Mead, 2014) rare museum specimens.

Furthermore, CT-based approaches allow for the quantification of complex three-dimensional structures, permitting geometrical comparisons of size and shape throughout ontogeny. Here we apply three-dimensional landmark-based geometric morphometrics to CT models of juvenile and adult *C. leucura* crania to describe ontogenetic changes in cranial osteology, test for sexual dimorphism in cranial shape, and determine the role of allometry (the scaling of biological shape with size) in generating intraspecific cranial variation.

Material and Methods

Thirty-nine individuals of *Cynisca leucura* (Duméril and Bibron, 1839) were collected for ecological studies in the rainy season of 2006 near the village of Batia (10°54'00.9'' N, 1°28'43.71'' E, 318 m a.s.l.), close to the Pendjari Biosphere Reserve in north-western Benin. The area has a dry Sudanian climate, with the mean annual precipitation (1000 mm) falling between late May and early October. The landscape is characterised by a mixture of different savanna types, fields, and fallows (Nago et al., 2006; Fig. 1). Specimens were anesthetized in a chlorobutanol solution and thereafter preserved in either 75% ethanol or 8% formalin.

Adult snout-vent lengths of *C. leucura* individuals ranged from 124–223 mm; juveniles were smaller than 100 mm (Table 1). Sex was determined by presence or absence of ovaries, and males were confirmed under a dissecting microscope by the presence of hemipenes. In addition to size, adults and juveniles were distinguished by sexual maturity:

in some juveniles the sex was unclear even after dissection, because gonads were only vaguely visible and did not show clear differentiation. A male or female juvenile was identified when sex was visible, but was clearly below the respective maximum size. Our final data set consisted of 26 adults (13 females, 13 males) and 13 juveniles (3 females, 6 males, 4 unknown). Specimens were deposited in the herpetological collection of the Museum für Naturkunde Berlin, Germany (see Table 1 for voucher numbers). All aspects of the research adhered to the legal requirements of Benin, and to the guidelines compiled by the American Society of Ichthyologists and Herpetologists (ASIH), The Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR).

X-ray Computed Tomography and Cranial Landmarks

Individuals were CT scanned at the Museum für Naturkunde Berlin in a Phoenix|x-ray Nanotom (GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany) equipped with a 180 kV nanofocus tube and a tungsten target. Prior to scanning, whole preserved animals were removed from ethanol, wrapped in plastic bubble wrap, and transferred to a dry 15ml centrifuge tube. All scans were made at 75 kV and 180 μ A for 750 ms over 800 projections, resulting in a final voxel size of approximately 7 μ m. Reconstructions were performed in Datos|x-reconstruction software (GE Sensing & Inspection Technologies GmbH phoenix|x-ray) and three-dimensional cranial surface models were isolated and extracted in VGStudio Max 2.1 (Volume Graphics, Heidelberg, Germany).

Cranial landmarks were chosen to describe biologically meaningful features, such as contacts between bones, tips of processes, and orbital and nasal cavities. Landmarks were also required to be ontogenetically stable, such that they could be located on both

juveniles and adults. In total, twenty-seven landmarks were digitally placed in Landmark Editor (Institute of Data Analysis and Visualization) on the dorsal, lateral and ventral surfaces of the cranium (Fig. 2).

Geometric Morphometric Analyses

To extract geometric shape information from the landmark coordinates, landmark data were subjected to a Generalized Procrustes fit. The resulting Procrustes coordinates represent the symmetric component of variation after scaling, translating and rotating all individuals to a common consensus, and were used as shape variables in all analyses. Centroid size, calculated as the square root of the sum of squared distances of landmarks to the center of their configuration, was used to describe individual size.

Variation in cranial shape was explored with a number of statistical tests. We first tested for evidence of group structure in our data using Procrustes analysis of variance (ANOVA), with age (juvenile vs. adult), sex, and their interaction as factors. Tests of allometric differentiation during ontogeny were made using multivariate analysis of covariance (MANCOVA), with log-transformed centroid size (logCS) as the covariate, and age, sex, and their interaction as factors. Ontogenetic allometry, describing the covariation of cranial shape and size during development, was visualized by multivariate regression of the Procrustes coordinates on logCS. Dominant axes of shape variation during ontogeny were identified by principal component analysis (PCA), performed on the covariance matrix of the Procrustes coordinates.

All tests were performed in MorphoJ (Klingenberg, 2011), except for the ANOVA/MANCOVA which were estimated in the Geomorph package v.2.1.5 of R

(Adams et al., 2015). For the latter tests, statistical significance ($\alpha=0.05$) was determined by a random residual permutation procedure of 10000 iterations. Warped surface models of crania used to illustrate the extremes of shape variation were made in Landmark Editor after exporting associated landmark coordinate files from MorphoJ.

Results

Cranial shape differed significantly among juveniles and adults, but not between males and females nor for any combination of age and sex (Table 2A). Similar patterns were observed for allometric variation, with juveniles and adults exhibiting significant differences in ontogenetic trajectories but males and females of different growth stages sharing a common allometric slope (Table 2B). Given the lack of sexual dimorphism in cranial shape, males and females were pooled in all further analyses.

Cranial shape varied strongly with size (regression of Procrustes shape coordinates on logCS for all individuals: $R^2=0.45$, $p<0.0001$), with juveniles growing more allometrically than adults as illustrated by a slightly steeper ontogenetic slope (Fig. 3A). Size-dependent shape changes mainly affect the postorbital region of the cranium, which becomes more slender and dorsoventrally compressed in adults (Fig. 3B; see also Supporting Information, Supplementary Figs. 1, 2). During growth, the parietal, which is smoothly dome-shaped in juveniles, becomes **mediolaterally constricted posterior to the frontoparietal suture, thus forming the narrowest point of the adult skull**. The apical process of the parietal, which is not yet formed in juveniles, becomes the tallest point of the adult cranium. **Along the midline length of the parietal bone, the sagittal crest appears as a**

slightly raised ridge in adults but remains relatively weakly developed posterior to the apical process, particularly compared to other amphisbaenians in which the sagittal crest comprises nearly one-third of the total parietal height (e.g., *Amphisbaena alba*, Montero and Gans 1999; Maisano et al., 2006). The degree of interdigitation between many of the anterior dorsal bones also increases during growth, with the anterolateral processes of the parietal and frontals shifting inwards medially and extending further anteriorly. Ventrally, the pterygoids lengthen posteriorly and swing inwards towards the midline of the skull.

In contrast to the bones of the snout, which become more tightly interconnected with increasing size, many of the lateral and ventral bones of the cranium remain loosely separated in the adult, with visible gaps between the prefrontal, orbitosphenoid, maxilla, vomer, palatine, ectopterygoid, pterygoid and basioccipital (Fig. 3B; Supporting Information, Supplementary Figs. 1, 2). One exception is the contact between the parietal and otooccipital complex, which appears nearly fused or strongly overlapping in juveniles, but is separated by a relatively simple open suture in adults.

Significant differences in cranial ontogeny were also observed in the PCA (Fig. 4), in which the first axis accounts for nearly half (47%) of the total variance. Adults fall almost exclusively on the negative side of PC1, whereas juveniles are on the positive side, reflecting the same ontogenetic changes described above. Positive movement along the second PC, accounting for another 10% of the total variance, describes a wider, more bulbous snout with an anteriorly shortened premaxillary process and a taller, more dome-shaped parietal.

Discussion

As descendants of fully-limbed terrestrial lizards (Townsend et al., 2004; Vidal et al., 2008; Longrich et al., 2015), amphisbaenians have undergone enormous transformations during their evolutionary history. Adaptations to a fossorial lifestyle required extensive changes in body shape, life history, and behavior, including multiple cranial specializations for head-first digging (Müller et al., 2011). Although cranial osteology provides the majority of morphological characters used in squamate systematics (e.g., Conrad, 2008; Gauthier et al., 2012), little is known about intraspecific variation in the amphisbaenian skull, potentially limiting our understanding of evolutionary relationships and ecological interactions. Here we applied geometric morphometric analyses to three-dimensional cranial models of the round-headed African amphisbaenid *Cynisca leucura* to identify morphological changes associated with age and sex, and to determine how allometry, or the scaling relationship between size and shape, affects adult cranial variation.

Based on our results, we found that cranial osteology of *C. leucura* is highly conserved, with the majority of shape changes occurring during individual growth. Intraspecific variation was strongly correlated with size change from juveniles to adults, indicating a dominant role of ontogenetic allometry in determining cranial shape. Ontogenetic allometry refers to covariation between size and shape during growth, arising as a consequence of developmental or functional interactions (Klingenberg, 2008; Pélabon et al., 2014). In *C. leucura*, ontogenetic changes were mainly seen in the postorbital region of the skull, which became longer and thinner as animals increased in size, while snout shape remained relatively unchanged (Figs. 3B, 4; Supporting Information, Supplementary Figs. 1, 2). This decoupling of allometric growth patterns among regions may reflect

different functional demands on the front versus back of the head, giving rise to modules or sets of highly correlated traits that evolve (semi-)independently (Klingenberg, 2008). Since the amphisbaenian snout is the primary point of contact with substrate during head-first burrowing (Gans, 1978), preservation of snout shape during growth may be critical to maintaining adequate digging performance over an individual's lifetime. Increasing interdigitation among the anterior dermal roofing bones (e.g., parietal, frontals and nasals) likely supports this activity, enhancing stability of the snout as the animal excavates progressively larger burrows.

In contrast to the dorsal cranial bones of *C. leucura* which maintain close, often increasing, contact throughout development, the bones of the lateral and ventral sides remain only loosely connected, as observed in the largest (and potentially oldest) male (Fig. 3B; Supporting Information, Supplementary Fig. 2). In life, these sutures are filled with broad bands of connective tissue that prevent slippage between bones, permitting substantial compression and torsional loading of the skull during burrow excavation (Montero and Gans, 1999). Not all amphisbaenians, however, exhibit this pattern, some possessing more rigid palatal regions formed by the tightly compressed premaxilla, maxillae, vomers, palatines, ectopyergoids and pterygoids (e.g., the shovel-headed *Rhineura floridana*; The Deep Scaly Project, 2008, but also the round-headed *Amphisbaena alba*; Montero and Gans, 1999). Determining if the extent of palatal closure reflects phylogenetic affinities or functional considerations will require additional data from a broad range of species and habitats, since the forces exerted by different substrate types and burrowing techniques are expected to affect cranial shape (Gans, 1960). In blunt round-headed forms like *Cynisca* and the majority of amphisbaenian species, the head is used as a

battering ram to penetrate the soil surface, where it is then pushed in different directions to compress the soil against the sides of the burrow (Gans, 1968). Although meta- and mesokinetik movements appear improbable in *C. leucura*, our results suggest that the ventral portion of the cranium may retain some flexibility, possibly as a protective mechanism for withstanding repeated pressure on the skull during this activity. Palatal mobility (i.e., displacement or sliding) has also been associated with feeding strategy in some fossorial lizards (e.g., *Lialis*; Hutchinson, 1993), although whether this pertains to amphisbaenians is still unknown.

Despite individual shape variation, no discernable differences between males and females were observed in morphospace occupation (Fig. 4). In addition to body length, head shape is one of the most consistently dimorphic traits in lizards (Olsson et al., 2002), with larger male heads associated with enhanced success in male-male competition (e.g., Vitt and Cooper, 1985; Anderson and Vitt, 1990) and increased bite force and prey handling efficiency (Verwaijen et al., 2002). Evidence of sexual dimorphism in amphisbaenians is mixed: males can be larger than females (*Amphisbaena nigricaudata*, Souza e Lima, 2014; *Leposternon polystegum*, Gomes et al., 2009), females can be larger than males (*Anops kingii*, Vega, 2001; *Zygaspis quadrifrons*, Webb et al., 2000), sexes can have similar body lengths but larger heads in one (males in *Trogonophis wiegmanni*, Martín et al., 2012; *Blanus cinereus*, Gil et al., 1993; *Leposternon microcephalum* and *L. wuchereri*, Filogonio et al., 2009; and females in *Monopeltis anchietae*, Webb et al., 2000), or sexes can be the same in both body length and head size (*Bipes biporus*, Kearney, 2003b; *Amphisbaena ibijara*, Gomes et al., 2009). We found no evidence of sexual dimorphism in *C. leucura*, either during ontogeny or among adults. Whether this reflects the absence of

sexual selection pressures (e.g., competition for mates, female fecundity) in this and other species is still unknown, [since](#) reproductive behavior of amphisbaenians has rarely been observed (Souza e Lima, 2014; but see Cooper et al., 1994, Filogonio et al., 2009).

Other possible explanations for cranial homogeneity in *C. leucura* involve ecological factors, including the absence of individual variation in microhabitat or diet. [Whereas](#) surface-dwelling reptiles commonly exploit different microhabitats based on individual preferences for thermoregulation, foraging, reproduction, or predator avoidance, burrowing lizards may be more constrained in their selection of optimal habitats (Martín et al., 1991, 2013). Loss or reduction of limbs presumably limits [abilities of amphisbaenians](#) to move between locations (Vidal et al., 2008), and their thigmothermic nature (i.e., gaining heat through conduction) means that they remain near rocks or leaf litter to maintain preferred body temperatures during cooler periods, but stay buried deep in the ground in summer to avoid overheating (Civantos et al., 2003). Physical and chemical properties of the soil in which they live may also restrict habitat use. For example, sandy soils are easier to excavate, whereas [clay-rich](#) soils are more compact when overly wet or dry (Gans, 1974; Martín et al., 1991). Ecological requirements likely limit food availability as well: in a survey of literature on amphisbaenian stomach contents, Kearney (2003b) found that most species exhibited a high degree of consistency in diet, both in terms of prey items and order of importance. Although no information is available on the ecological [tolerances](#) of *C. leucura*, its habitat suggests some influence of variation in radiation, temperature and precipitation. How these climatic variables affect fossorial squamates, or if subterranean environments are buffered from such changes, remains to be tested. Interestingly, a close congener, *Cynisca rouxae*, occurring in similar West African savanna habitats as *C.*

leucura, was only found during the rainy season, indicating movement into deeper ground during the dry season (Rödel and Grabow, 1996).

Regardless of potential extrinsic selective forces, our results show that ontogenetic allometry, in particular its influence on snout shape, acts as a developmental constraint in *C. leucura*. Developmental constraints are mechanisms that limit or channel variation in adult phenotypes, however allometries themselves may represent optimal scaling relationships between traits, shaped by natural selection (Klingenberg, 2010; Pélabon et al., 2014). Given the fossorial habits of *C. leucura*, we hypothesize that ontogenetic allometry is under strong stabilizing selection to maintain adequate cranial proportions for head-first digging, thereby constraining the ability of individuals to respond to the various selection pressures described above. Previous authors showed that small changes in amphisbaenian head shape can affect burrowing performance, with longer, narrower heads burrowing faster (López et al., 1997; Navas et al., 2004). Therefore, differing allometric growth patterns observed between pre- and postorbital regions may reflect biomechanical trade-offs for this behavior, by which the snout becomes more highly reinforced as the animal digs larger burrows, while the back of the head becomes longer and thinner to maximize burrowing efficiency. This scenario would be in agreement with the physiological model of head-first digging by Navas et al. (2004), showing that as the amphisbaenian body grows, the work required to compress soil increases exponentially, thus imposing great physical stress on the skull.

In soil types where burrowing requires less force, for example in soft, sandy soils, selection on allometric relationships during growth may be weakened, allowing changes to the ancestral ontogeny and subsequent morphological traits. Such evolutionary dissociation

between size and shape, known as heterochrony, alters the timing and rate of developmental events, thus giving rise to new descendent forms. Similar heterochronic processes may be responsible for the evolution of the other amphisbaenian cranial shapes as well, which may themselves represent adaptive compromises between functional limitations and digging performance for their respective burrowing techniques.

Comparisons of ontogenetic trajectories among species in different habitats may reveal if cranial growth patterns are associated with ecological niche, thus explaining the high degree of convergence observed across the amphisbaenian tree (e.g., Kearney and Stuart, 2004). Mapping those patterns onto a dated molecular phylogeny could also indicate where and when heterochronic shifts in ontogeny occur, and if those transitions coincide with other morphological transformations (e.g., loss of the postorbital rim, Kearney 2003a; pectoral and pelvic girdle reduction, Kearney, 2002) or ancient dispersal events (e.g., Vidal et al., 2008; Longrich et al., 2015). The relative stability of cranial shapes throughout the amphisbaenian fossil record (from the early Eocene on; Hipsley and Müller, 2014) suggests that despite the potential for heterochronic change, amphisbaenian cranial evolution is confined to a limited set of developmental pathways.

Conclusions

Intraspecific variation is a critical component of biodiversity, since it is the variation *within* species that ultimately gives rise to the differences *between* them. While some taxa lie on a phenotypic continuum along which intraspecific variation can overlap or blur interspecific distances, others form more discrete phenotypic clusters or morphotypes. Understanding why species vary in this regard and how levels of variation interact in the face of selection

is a central goal of evolutionary biology, both for identifying the factors controlling diversification, and for defining species as taxonomically relevant units (i.e., the phenetic species concept).

Despite the recognized role of intraspecific variation in shaping evolutionary dynamics, detailed studies of skeletal morphology in squamate reptiles are commonly based on single specimens (the $n = 1$ phenomenon; Bell and Mead, 2014). Here we show that in addition to increasing sample size, the inclusion of ontogenetic series in studies of individual taxa can provide important insights into patterns of skeletal variation within species, as well as the extent to which they are influenced by internal (phylogenetic) or external (ecological) factors. The unresolved paradox between the conserved cranial ontogeny of *Cynisca leucura* and homoplasy of head shapes throughout amphisbaenian history indicates a still vital role for developmental studies in this group, since evolutionary change in morphological features requires change in the developmental processes that produce them.

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Author contributions

M-OR collected specimens, JM and CAH conceived the project, MR CT scanned specimens and created 3D cranial models, CAH performed cranial landmarking and analyses and wrote the manuscript. All authors read, commented on and approved the final version.

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Figure legends

Figure 1. Taxon sampling. Head of *Cynisca leucura* (ZMB 79353) in (A) dorsal, (B) lateral and (C) ventral views, showing the distinct round-headed morphology. (D) *C. leucura* in life (photo courtesy of J. Penner) and (E) its habitat in northern Benin.

Figure 2. Locations of 27 cranial landmarks used in geometric morphometric analyses.

Figure 3. Cranial ontogeny of *Cynisca leucura*. (A) Ontogenetic allometry accounts for 45% of cranial shape variation during growth, with juveniles and adults exhibiting significantly different allometric slopes. Symbols: Open=female, shaded=male, circle=adult, triangle=juvenile, X=juvenile of unknown sex. (B) Dorsal, lateral and ventral views of juvenile (left) and adult (right) specimens of *C. leucura*, illustrating small and large size extremes. Abbreviations: app, apical process of parietal; bo, basioccipital; ec, ectopterygoid; f, frontal; m, maxilla; n, nasal; ooc, otooccipital complex; os, orbitosphenoid; pal, palatine; p, parietal; pf, prefrontal; pm, premaxilla; pt, pterygoid; v, vomer.

Figure 4. Major axes of cranial shape variation in *Cynisca leucura*. Warped cranial surfaces represent positive and negative shape extremes for the first two PC axes. Symbols: Open=female, shaded=male, circle=adult, triangle=juvenile, X=juvenile of unknown sex.

Table 1. *Cynisca leucura* specimens investigated in the present study, deposited at the Museum für Naturkunde Berlin (ZMB). Individual age, sex, and snout-vent length (SVL) are given.

Voucher number	age	sex	SVL (mm)
ZMB 79376	juvenile	F	65.32
ZMB 79359	juvenile	F	74.33
ZMB 79374	juvenile	M	77.02
ZMB 79370	juvenile	-	77.23
ZMB 79375	juvenile	M	77.86
ZMB 79348	juvenile	F	77.99
ZMB 79371	juvenile	M	78.2
ZMB 79373	juvenile	M	81.72
ZMB 79369	juvenile	-	82.98
ZMB 79358	juvenile	M	84.7
ZMB 79368	juvenile	-	88.85
ZMB 79357	juvenile	-	92.33
ZMB 79372	juvenile	M	96.57
ZMB 79347	adult	M	124.2
ZMB 79093	adult	M	146.51
ZMB 79362	adult	F	146.74
ZMB 79356	adult	F	155.12
ZMB 79350	adult	F	163.31
ZMB 79352	adult	M	165.36
ZMB 79349	adult	F	167.58
ZMB 79367	adult	M	174.82
ZMB 79351	adult	M	177.31
ZMB 79341	adult	F	180.64
ZMB 79343	adult	M	183.26
ZMB 79344	adult	F	183.84
ZMB 79354	adult	M	185.32
ZMB 79342	adult	F	186.4
ZMB 79379	adult	F	186.74
ZMB 79345	adult	M	187.66
ZMB 79365	adult	M	193.78
ZMB 79361	adult	F	197.27
ZMB 79363	adult	F	199.69
ZMB 79360	adult	M	202.24
ZMB 79366	adult	M	208.93
ZMB 79346	adult	F	213.64
ZMB 79378	adult	F	214.1
ZMB 79355	adult	F	217.2
ZMB 79377	adult	M	221.42
ZMB 79353	adult	M	222.62

Table 2. *Cynisca leucura* cranial shape variation. (A) Procrustes ANOVA results of cranial shape variation among juveniles and adults (age), males and females (sex), and their interaction (age×sex). (B) MANCOVA results of cranial shape allometry with size (logCS=log-transformed centroid size), and its variation with age (logCS×age), sex (logCS×sex), and their interaction (logCS×(age×sex)).

	df	SS	MS	R ²	F	Z	P
(A) Cranial shape variation							
Age	1	0.0245	0.0245	0.3882	23.477	12.275	0.0001
Sex	1	0.0009	0.0009	0.0175	0.5878	0.4971	0.7321
Age×sex	1	0.001	0.001	0.0188	0.9956	0.9989	0.3682
(B) Cranial shape allometry							
logCS	1	0.0285	0.0285	0.4523	30.553	14.051	0.0001
logCS×age	1	0.0017	0.0017	0.0267	1.8625	1.8068	0.0194
logCS×sex	1	0.0009	0.0009	0.0174	1.0477	1.0583	0.3214
logCS×(age×sex)	1	0.0011	0.0011	0.0208	1.2787	1.4536	0.0728

Figure 1. Taxon sampling. Head of *Cynisca leucura* (ZMB 79353) in (A) dorsal, (B) lateral and (C) ventral views, showing the distinct round-headed morphology. (D) *C. leucura* in life (photo courtesy of J. Penner) and (E) its habitat in northern Benin.
190x127mm (150 x 150 DPI)

Accept

Figure 2. Locations of 27 cranial landmarks used in geometric morphometric analyses.
81x98mm (300 x 300 DPI)

A

Figure 3. Cranial ontogeny of *Cynisca leucura*. (A) Ontogenetic allometry accounts for 45% of cranial shape variation during growth, with juveniles and adults exhibiting significantly different allometric slopes.

Symbols: Open=female, shaded=male, circle=adult, triangle=juvenile, X=juvenile of unknown sex. (B) Dorsal, lateral and ventral views of juvenile (left) and adult (right) specimens of *C. leucura*, illustrating small and large size extremes. Abbreviations: app, apical process of parietal; bo, basioccipital; ec, ectopterygoid; f, frontal; m, maxilla; n, nasal; ooc, otooccipital complex; os, orbitosphenoid; pal, palatine; p, parietal; pf, prefrontal; pm, premaxilla; pt, pterygoid; v, vomer.

150x142mm (300 x 300 DPI)

A

Figure 4. Major axes of cranial shape variation in *Cynisca leucura*. Warped cranial surfaces represent positive and negative shape extremes for the first two PC axes. Symbols: Open=female, shaded=male, circle=adult, triangle=juvenile, X=juvenile of unknown sex.
111x68mm (300 x 300 DPI)

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Ontogenetic allometry constrains cranial shape of the head-first burrowing worm lizard *Cynisca leucura* (Squamata: Amphisbaenidae)

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**Ontogenetic allometry constrains cranial shape of the head-first burrowing worm
lizard *Cynisca leucura* (Squamata: Amphisbaenidae)**

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Short title: *Cynisca leucura* cranial ontogeny

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Abstract: Amphisbaenians are fossorial, predominantly limbless squamate reptiles with distinct cranial shapes corresponding to specific burrowing behaviors. Due to their cryptic lifestyles and the scarcity of museum specimens, little is known of their intraspecific variation, particularly regarding cranial osteology. This represents a critical lack of information, since the majority of morphological investigations of squamate relationships are based on cranial characters. We investigated cranial variation in the West African Coast Worm Lizard *Cynisca leucura*, a round-headed member of the ~~family~~ Amphisbaenidae. Using geometric morphometric analyses of three-dimensional computed tomographic scans, we found that cranial osteology of *C. leucura* is highly conserved, with the majority of shape changes occurring during growth as the cranium becomes more slender and elongate, accompanied by increasing interdigitation among the dermal roofing bones. Elements of the ventral portion of the cranium remain loosely connected in adults, possibly as a protective mechanism against repeated compression and torsion during burrow excavation. Intraspecific variation was strongly correlated with size change from juveniles to adults, indicating a dominant role of ontogenetic allometry in determining cranial shape. We found no evidence of sexual dimorphism, either during growth or among adults. Given the fossorial habits of *C. leucura*, we hypothesize that cranial allometry is under strong stabilizing selection to maintain adequate proportions for head-first digging, thereby constraining the ability of individuals to respond to differing selection pressures, including sexual selection and variation in diet or microhabitat. For species in which digging imposes less mechanical stress (e.g., in softer sand), allometric associations during growth may be weakened, allowing changes to the ontogenetic trajectory and subsequent morphological traits. Such developmental dissociation between size and shape, known as heterochrony,

may also be implicit in the evolution of the other amphisbaenian cranial shapes (shovel, spade and keel), which may themselves be functionally adapted for their respective burrowing techniques.

Key words: ~~Amphisbaenia, *Cynisca leucura*, ontogeny, allometry, cranial shape~~

Accepted Article

Introduction

Amphisbaenians, or worm lizards, comprise nearly 200 species of small fossorial reptiles inhabiting tropical and semitropical regions of the world, of which all but three are limbless (Kearney, 2003a). Since separating from their closest relatives, lacertid lizards (Townsend et al., 2004; Vidal et al., 2008; Müller et al., 2011; Wiens et al., 2012), amphisbaenians evolved numerous adaptations for underground locomotion, including four distinct cranial shapes (shovel, spade, keel, and round) each associated with a particular head-first burrowing technique (Gans, 1974). Although some studies place two of the cranial morphotypes in monophyletic groups (shovel and keel-headed; Vanzolini, 1951; Kearney, 2003a), the high degree of conflict between morphological and molecular data has made phylogenetic reconstructions problematic. For example, morphological analyses recover the round-headed *Bipes* (the only genus with (fore)limbs) as most primitive (Gans, 1978; Kearney, 2003a), while molecular evidence places the shovel-headed Rhineuridae at the base of the tree with *Bipes* nested deep within the other limbless taxa (Kearney and Stuart, 2004; Macey et al., 2004; Vidal et al., 2008; Mott and Vieites, 2009). Which of these hypotheses, if any, is correct has significant consequences on our interpretations of character homology and limb loss (or gain).

Regardless of exact relationships, the Amphisbaenidae represents the most diverse (>170 species) and widespread amphisbaenian family, distributed across the Caribbean, sub-Saharan Africa, and South America (Gans, 2005). It is also the most disparate, with extant forms encompassing three of the four cranial morphologies (Kearney, 2003a). The

fourth cranial shape (spade-headed) is found in the Miocene African amphisbaenid *Listromycter leakeyi* (Charig and Gans, 1990).

Despite their relatively high diversity, few attempts have been made to characterize variation in amphisbaenid species in detail, particularly in terms of morphology but also regarding life history, behavior, diet, and geographical range (but see Montero et al., 1999; Vega, 2001; Ballestrin and Capellari, 2001; Vanzolini, 2002). In fact, many species are only known from the type or type series (see Gans, 2005), making estimates of intraspecific variation difficult. This is particularly true for features of the skull, which are often the focus of systematic investigations because they show marked interfamilial and intergeneric differences (Gans, 1978). Considering the high degree of variation reported for some conspecifics even from the same locality (e.g., one or two head shields among individuals of *Monopeltis guentheri*; Loveridge, 1941), understanding the range of morphological variation in amphisbaenid (and amphisbaenian) species would greatly expand our knowledge of this enigmatic group, and likely contribute to better supported phylogenetic hypotheses.

In this study, we examine intraspecific variation in the West African Coast Worm Lizard *Cynisca leucura*, a relatively small (<250 mm snout-vent length) round-headed member of the Amphisbaenidae. We focus on variation in cranial osteology using high-resolution X-ray computed tomography (CT), a non-destructive technique for visualizing internal anatomy of whole specimens. CT is particularly suitable for investigations of the amphisbaenian skull, since: 1) they are typically small (the skull of this species measures <1 cm in length); 2) they comprise overlapping and tightly compressed bones that are difficult to examine through traditional preparation; and 3) it circumvents the need to

disarticulate (but not destroy; see Bell and Mead, 2014) rare museum specimens.

Furthermore, CT-based approaches allow for the quantification of complex three-dimensional structures, permitting geometrical comparisons of size and shape throughout ontogeny. Here we apply three-dimensional landmark-based geometric morphometrics to CT models of juvenile and adult *C. leucura* crania to describe ontogenetic changes in cranial osteology, test for sexual dimorphism in cranial shape, and determine the role of allometry (the scaling of biological shape with size) in generating intraspecific cranial variation.

Material and Methods

Thirty-nine individuals of *Cynisca leucura* (Duméril and Bibron, 1839) were collected for ecological studies in the rainy season of 2006 near the village of Batia (10°54'00.9'' N, 1°28'43.71'' E, 318 m a.s.l.), close to the Pendjari Biosphere Reserve in north-western Benin. The area has a dry Sudanian climate, with the mean annual precipitation (1000 mm) falling between late May and early October. The landscape is characterised by a mixture of different savanna types, fields, and fallows (Nago et al., 2006; Fig. 1). Specimens were anesthetized in a chlorobutanol solution and thereafter preserved in either 75% ethanol or 8% formalin.

Adult snout-vent lengths of *C. leucura* individuals ranged from 124–223 mm; juveniles were smaller than 100 mm (Table 1). Sex was determined by presence or absence of ovaries, and males were confirmed under a dissecting microscope by the presence of hemipenes. In addition to size, adults and juveniles were distinguished by sexual maturity:

in some juveniles the sex was unclear even after dissection, because gonads were only vaguely visible and did not show clear differentiation. A male or female juvenile was identified when sex was visible, but was clearly below the respective maximum size. Our final data set consisted of 26 adults (13 females, 13 males) and 13 juveniles (3 females, 6 males, 4 unknown). Specimens were deposited in the herpetological collection of the Museum für Naturkunde Berlin, Germany (see Table 1 for voucher numbers). All aspects of the research adhered to the legal requirements of Benin, and to the guidelines compiled by the American Society of Ichthyologists and Herpetologists (ASIH), The Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR).

X-ray Computed Tomography and Cranial Landmarks

Individuals were CT-scanned at the Museum für Naturkunde Berlin in a Phoenix|x-ray Nanotom (GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany) equipped with a 180 kV nanofocus tube and a tungsten target. Prior to scanning, whole preserved animals were removed from ethanol, wrapped in plastic bubble wrap, and transferred to a dry 15ml centrifuge tube. All scans were made at 75 kV and 180 μ A for 750 ms over 800 projections, resulting in a final voxel size of approximately 7 μ m. Reconstructions were performed in Datos|x-reconstruction software (GE Sensing & Inspection Technologies GmbH phoenix|x-ray) and three-dimensional cranial surface models were isolated and extracted in VGStudio Max 2.1 (Volume Graphics, Heidelberg, Germany).

Cranial landmarks were chosen to describe biologically meaningful features, such as contacts between bones, tips of processes, and orbital and nasal cavities. Landmarks were also required to be ontogenetically stable, such that they could be located on both

juveniles and adults. In total, twenty-seven landmarks were digitally placed in Landmark Editor (Institute of Data Analysis and Visualization) on the dorsal, lateral and ventral surfaces of the cranium (Fig. 2).

Geometric Morphometric Analyses

To extract geometric shape information from the landmark coordinates, landmark data were subjected to a Generalized Procrustes fit. The resulting Procrustes coordinates represent the symmetric component of variation after scaling, translating and rotating all individuals to a common consensus, and were used as shape variables in all analyses. Centroid size, calculated as the square root of the sum of squared distances of landmarks to the center of their configuration, was used to describe individual size.

Variation in cranial shape was explored with a number of statistical tests. We first tested for evidence of group structure in our data using Procrustes analysis of variance (ANOVA), with age (juvenile vs. adult), sex, and their interaction as factors. Tests of allometric differentiation during ontogeny were made using multivariate analysis of covariance (MANCOVA), with log-transformed centroid size (logCS) as the covariate, and age, sex, and their interaction as factors. Ontogenetic allometry, describing the covariation of cranial shape and size during development, was visualized by multivariate regression of the Procrustes coordinates on logCS. Dominant axes of shape variation during ontogeny were identified by principal component analysis (PCA), performed on the covariance matrix of the Procrustes coordinates.

All tests were performed in MorphoJ (Klingenberg, 2011), except for the ANOVA/MANCOVA which were estimated in the Geomorph package v.2.1.5 of R

(Adams et al., 2015). For the latter tests, statistical significance ($\alpha=0.05$) was determined by a random residual permutation procedure of 10000 iterations. Warped surface models of crania used to illustrate the extremes of shape variation were made in Landmark Editor after exporting associated landmark coordinate files from MorphoJ.

Results

Cranial shape differed significantly among juveniles and adults, but not between males and females nor for any combination of age and sex (Table 2A). Similar patterns were observed for allometric variation, with juveniles and adults exhibiting significant differences in ontogenetic trajectories but males and females of different growth stages sharing a common allometric slope (Table 2B). Given the lack of sexual dimorphism in cranial shape, males and females were pooled in all further analyses.

Cranial shape varied strongly with size (regression of Procrustes shape coordinates on logCS for all individuals: $R^2=0.45$, $p<0.0001$), with juveniles growing more allometrically than adults as illustrated by a slightly steeper ontogenetic slope (Fig. 3A). Size-dependent shape changes mainly affect the postorbital region of the cranium, which becomes more slender and dorsoventrally compressed in adults (Fig. 3B; see also [Supporting Information, Supplementary Figs. 1, 2](#)). During growth, the parietal, which is smoothly dome-shaped in juveniles, becomes [mediolaterally constricted posterior to the frontoparietal suture, thus forming the narrowest point of the adult skull](#). The apical process of the parietal, which is not yet formed in juveniles, becomes the tallest point of the adult cranium. [Along the midline length of the parietal bone, the sagittal crest appears as a](#)

slightly raised ridge in adults but remains relatively weakly developed posterior to the apical process, particularly compared to other amphisbaenians in which the sagittal crest comprises nearly one-third of the total parietal height (e.g., *Amphisbaena alba*, Montero and Gans 1999; Maisano et al., 2006). The degree of interdigitation between many of the anterior dorsal bones also increases during growth, with the anterolateral processes of the parietal and frontals shifting inwards medially and extending further anteriorly. Ventrally, the pterygoids lengthen posteriorly and swing inwards towards the midline of the skull.

In contrast to the bones of the snout, which become more tightly interconnected with increasing size, many of the lateral and ventral bones of the cranium remain loosely separated in the adult, with visible gaps between the prefrontal, orbitosphenoid, maxilla, vomer, palatine, ectopterygoid, pterygoid and basioccipital (Fig. 3B; [Supporting Information, Supplementary Figs. 1, 2](#)). One exception is the contact between the parietal and otooccipital complex, which appears nearly fused or strongly overlapping in juveniles, but is separated by a relatively simple open suture in adults.

Significant differences in cranial ontogeny were also observed in the PCA (Fig. 4), in which the first axis accounts for nearly half (47%) of the total variance. Adults fall almost exclusively on the negative side of PC1, whereas juveniles are on the positive side, reflecting the same ontogenetic changes described above. Positive movement along the second PC, accounting for another 10% of the total variance, describes a wider, more bulbous snout with an anteriorly shortened premaxillary process and a taller, more dome-shaped parietal.

Discussion

As descendants of fully-limbed terrestrial lizards (Townsend et al., 2004; Vidal et al., 2008; Longrich et al., 2015), amphisbaenians have undergone enormous transformations during their evolutionary history. Adaptations to a fossorial lifestyle required extensive changes in body shape, life history, and behavior, including multiple cranial specializations for head-first digging (Müller et al., 2011). Although cranial osteology provides the majority of morphological characters used in squamate systematics (e.g., Conrad, 2008; Gauthier et al., 2012), little is known about intraspecific variation in the amphisbaenian skull, potentially limiting our understanding of evolutionary relationships and ecological interactions. Here we applied geometric morphometric analyses to three-dimensional cranial models of the round-headed African amphisbaenid *Cynisca leucura* to identify morphological changes associated with age and sex, and to determine how allometry, or the scaling relationship between size and shape, affects adult cranial variation.

Based on our results, we found that cranial osteology of *C. leucura* is highly conserved, with the majority of shape changes occurring during individual growth. Intraspecific variation was strongly correlated with size change from juveniles to adults, indicating a dominant role of ontogenetic allometry in determining cranial shape. Ontogenetic allometry refers to covariation between size and shape during growth, arising as a consequence of developmental or functional interactions (Klingenberg, 2008; Pélabon et al., 2014). In *C. leucura*, ontogenetic changes were mainly seen in the postorbital region of the skull, which became longer and thinner as animals increased in size, while snout shape remained relatively unchanged (Figs. 3B, 4; [Supporting Information, Supplementary Figs. 1, 2](#)). This decoupling of allometric growth patterns among regions may reflect

different functional demands on the front versus back of the head, giving rise to modules or sets of highly correlated traits that evolve (semi-)independently (Klingenberg, 2008). [Since](#) the amphisbaenian snout is the primary point of contact with substrate during head-first burrowing (Gans, 1978), preservation of snout shape during growth may be critical to maintaining [adequate](#) digging performance over an individual's lifetime. Increasing interdigitation among the anterior dermal roofing bones (e.g., parietal, frontals and nasals) likely [supports this activity](#), enhancing stability of the snout as the animal excavates progressively larger burrows.

In contrast to the dorsal cranial bones of *C. leucura* which maintain close, often increasing, contact throughout development, the bones of the lateral and ventral sides remain only loosely connected, as observed in the largest (and potentially oldest) male (Fig. 3B; [Supporting Information, Supplementary Fig. 2](#)). In life, these sutures are filled with broad bands of connective tissue that prevent slippage between bones, permitting substantial compression and torsional loading of the skull during burrow excavation (Montero and Gans, 1999). Not all amphisbaenians, however, exhibit this pattern, some possessing more rigid palatal regions formed by the tightly compressed premaxilla, maxillae, vomers, palatines, ectopyergoids and pterygoids (e.g., the shovel-headed *Rhineura floridana*; The Deep Scaly Project, 2008, but also the round-headed *Amphisbaena alba*; Montero and Gans, 1999). Determining if the extent of palatal closure reflects phylogenetic affinities or functional considerations will require additional data from a broad range of species and habitats, [since](#) the forces exerted by different substrate types and burrowing techniques are expected to affect cranial shape (Gans, 1960). In blunt round-headed forms like *Cynisca* and the majority of amphisbaenian species, the head is used as a

battering ram to penetrate the soil surface, where it is then pushed in different directions to compress the soil against the sides of the burrow (Gans, 1968). Although meta- and mesokinetically movements appear improbable in *C. leucura*, our results suggest that the ventral portion of the cranium may retain some flexibility, possibly as a protective mechanism for withstanding repeated pressure on the skull during this activity. Palatal mobility (i.e., displacement or sliding) has also been associated with feeding strategy in some fossorial lizards (e.g., *Lialis*; Hutchinson, 1993), although whether this pertains to amphisbaenians is still unknown.

Despite individual shape variation, no discernable differences between males and females were observed in morphospace occupation (Fig. 4). In addition to body length, head shape is one of the most consistently dimorphic traits in lizards (Olsson et al., 2002), with larger male heads associated with enhanced success in male-male competition (e.g., Vitt and Cooper, 1985; Anderson and Vitt, 1990) and increased bite force and prey handling efficiency (Verwaijen et al., 2002). Evidence of sexual dimorphism in amphisbaenians is mixed: males can be larger than females (*Amphisbaena nigricaudata*, Souza e Lima, 2014; *Leposternon polystegum*, Gomes et al., 2009), females can be larger than males (*Anops kingii*, Vega, 2001; *Zygaspis quadrifrons*, Webb et al., 2000), sexes can have similar body lengths but larger heads in one (males in *Trogonophis wiegmanni*, Martín et al., 2012; *Blanus cinereus*, Gil et al., 1993; *Leposternon microcephalum* and *L. wuchereri*, Filogonio et al., 2009; and females in *Monopeltis anchietae*, Webb et al., 2000), or sexes can be the same in both body length and head size (*Bipes biporus*, Kearney, 2003b; *Amphisbaena ibijara*, Gomes et al., 2009). We found no evidence of sexual dimorphism in *C. leucura*, either during ontogeny or among adults. Whether this reflects the absence of

sexual selection pressures (e.g., competition for mates, female fecundity) in this and other species is still unknown, [since](#) reproductive behavior of amphisbaenians has rarely been observed (Souza e Lima, 2014; but see Cooper et al., 1994, Filogonio et al., 2009).

Other possible explanations for cranial homogeneity in *C. leucura* involve ecological factors, including the absence of individual variation in microhabitat or diet. [Whereas](#) surface-dwelling [reptiles](#) commonly exploit different microhabitats based on individual preferences for thermoregulation, foraging, reproduction, or predator avoidance, burrowing lizards may be more constrained in their selection of optimal habitats (Martín et al., 1991, 2013). Loss or reduction of limbs presumably limits [abilities of amphisbaenians](#) to move between locations (Vidal et al., 2008), and their thigmothermic nature (i.e., gaining heat through conduction) means that they remain near rocks or leaf litter to maintain preferred body temperatures during cooler periods, but stay buried deep in the ground in summer to avoid overheating (Civantos et al., 2003). Physical and chemical properties of the soil in which they live may also restrict habitat use. For example, sandy soils are easier to excavate, whereas [clay-rich](#) soils are more compact when overly wet or dry (Gans, 1974; Martín et al., 1991). Ecological requirements likely limit food availability as well: in a survey of literature on amphisbaenian stomach contents, Kearney (2003b) found that most species exhibited a high degree of consistency in diet, both in terms of prey items and order of importance. Although no information is available on the ecological [tolerances](#) of *C. leucura*, its habitat suggests some influence of variation in radiation, temperature and precipitation. How these climatic variables affect fossorial squamates, or if subterranean environments are buffered from such changes, remains to be tested. Interestingly, a close congener, *Cynisca rouxae*, occurring in similar West African savanna habitats as *C.*

leucura, was only found during the rainy season, indicating movement into deeper ground during the dry season (Rödel and Grabow, 1996).

Regardless of potential extrinsic selective forces, our results show that ontogenetic allometry, in particular its influence on snout shape, acts as a developmental constraint in *C. leucura*. Developmental constraints are mechanisms that limit or channel variation in adult phenotypes, however allometries themselves may represent optimal scaling relationships between traits, shaped by natural selection (Klingenberg, 2010; Pélabon et al., 2014). Given the fossorial habits of *C. leucura*, we hypothesize that ontogenetic allometry is under strong stabilizing selection to maintain adequate cranial proportions for head-first digging, thereby constraining the ability of individuals to respond to the various selection pressures described above. Previous authors showed that small changes in amphisbaenian head shape can affect burrowing performance, with longer, narrower heads burrowing faster (López et al., 1997; Navas et al., 2004). Therefore, differing allometric growth patterns observed between pre- and postorbital regions may reflect biomechanical trade-offs for this behavior, by which the snout becomes more highly reinforced as the animal digs larger burrows, while the back of the head becomes longer and thinner to maximize burrowing efficiency. This scenario would be in agreement with the physiological model of head-first digging by Navas et al. (2004), showing that as the amphisbaenian body grows, the work required to compress soil increases exponentially, thus imposing great physical stress on the skull.

In soil types where burrowing requires less force, for example in soft, sandy soils, selection on allometric relationships during growth may be weakened, allowing changes to the ancestral ontogeny and subsequent morphological traits. Such evolutionary dissociation

between size and shape, known as heterochrony, alters the timing and rate of developmental events, thus giving rise to new descendent forms. Similar heterochronic processes may be responsible for the evolution of the other amphisbaenian cranial shapes as well, which may themselves represent adaptive compromises between functional limitations and digging performance for their respective burrowing techniques.

Comparisons of ontogenetic trajectories among species in different habitats may reveal if cranial growth patterns are associated with ecological niche, thus explaining the high degree of convergence observed across the amphisbaenian tree (e.g., Kearney and Stuart, 2004). Mapping those patterns onto a dated molecular phylogeny could also indicate where and when heterochronic shifts in ontogeny occur, and if those transitions coincide with other morphological transformations (e.g., loss of the postorbital rim, Kearney 2003a; pectoral and pelvic girdle reduction, Kearney, 2002) or ancient dispersal events (e.g., Vidal et al., 2008; Longrich et al., 2015). The relative stability of cranial shapes throughout the amphisbaenian fossil record (from the early Eocene on; Hipsley and Müller, 2014) suggests that despite the potential for heterochronic change, amphisbaenian cranial evolution is confined to a limited set of developmental pathways.

Conclusions

Intraspecific variation is a critical component of biodiversity, since it is the variation *within* species that ultimately gives rise to the differences *between* them. While some taxa lie on a phenotypic continuum along which intraspecific variation can overlap or blur interspecific distances, others form more discrete phenotypic clusters or morphotypes. Understanding why species vary in this regard and how levels of variation interact in the face of selection

is a central goal of evolutionary biology, both for identifying the factors controlling diversification, and for defining species as taxonomically relevant units (i.e., the phenetic species concept).

Despite the recognized role of intraspecific variation in shaping evolutionary dynamics, detailed studies of skeletal morphology in squamate reptiles are commonly based on single specimens (the $n = 1$ phenomenon; Bell and Mead, 2014). Here we show that in addition to increasing sample size, the inclusion of ontogenetic series in studies of individual taxa can provide important insights into patterns of skeletal variation within species, as well as the extent to which they are influenced by internal (phylogenetic) or external (ecological) factors. The unresolved paradox between the conserved cranial ontogeny of *Cynisca leucura* and homoplasy of head shapes throughout amphisbaenian history indicates a still vital role for developmental studies in this group, since evolutionary change in morphological features requires change in the developmental processes that produce them.

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Author contributions

M-OR collected specimens, JM and CAH conceived the project, MR CT scanned specimens and created 3D cranial models, CAH performed cranial landmarking and analyses and wrote the manuscript. All authors read, commented on and approved the final version.

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Figure legends

Figure 1. Taxon sampling. Head of *Cynisca leucura* (ZMB 79353) in (A) dorsal, (B) lateral and (C) ventral views, showing the distinct round-headed morphology. (D) *C. leucura* in life (photo courtesy of J. Penner) and (E) its habitat in northern Benin.

Figure 2. Locations of 27 cranial landmarks used in geometric morphometric analyses.

Figure 3. Cranial ontogeny of *Cynisca leucura*. (A) Ontogenetic allometry accounts for 45% of cranial shape variation during growth, with juveniles and adults exhibiting significantly different allometric slopes. Symbols: Open=female, shaded=male, circle=adult, triangle=juvenile, X=juvenile of unknown sex. (B) Dorsal, lateral and ventral views of juvenile (left) and adult (right) specimens of *C. leucura*, illustrating small and large size extremes. Abbreviations: app, apical process of parietal; bo, basioccipital; ec, ectopterygoid; f, frontal; m, maxilla; n, nasal; ooc, otooccipital complex; os, orbitosphenoid; pal, palatine; p, parietal; pf, prefrontal; pm, premaxilla; pt, pterygoid; v, vomer.

Figure 4. Major axes of cranial shape variation in *Cynisca leucura*. Warped cranial surfaces represent positive and negative shape extremes for the first two PC axes. Symbols: Open=female, shaded=male, circle=adult, triangle=juvenile, X=juvenile of unknown sex.

Table 1. *Cynisca leucura* specimens investigated in the present study, deposited at the Museum für Naturkunde Berlin (ZMB). Individual age, sex, and snout-vent length (SVL) are given.

Voucher number	age	sex	SVL (mm)
ZMB 79376	juvenile	F	65.32
ZMB 79359	juvenile	F	74.33
ZMB 79374	juvenile	M	77.02
ZMB 79370	juvenile	-	77.23
ZMB 79375	juvenile	M	77.86
ZMB 79348	juvenile	F	77.99
ZMB 79371	juvenile	M	78.2
ZMB 79373	juvenile	M	81.72
ZMB 79369	juvenile	-	82.98
ZMB 79358	juvenile	M	84.7
ZMB 79368	juvenile	-	88.85
ZMB 79357	juvenile	-	92.33
ZMB 79372	juvenile	M	96.57
ZMB 79347	adult	M	124.2
ZMB 79093	adult	M	146.51
ZMB 79362	adult	F	146.74
ZMB 79356	adult	F	155.12
ZMB 79350	adult	F	163.31
ZMB 79352	adult	M	165.36
ZMB 79349	adult	F	167.58
ZMB 79367	adult	M	174.82
ZMB 79351	adult	M	177.31
ZMB 79341	adult	F	180.64
ZMB 79343	adult	M	183.26
ZMB 79344	adult	F	183.84
ZMB 79354	adult	M	185.32
ZMB 79342	adult	F	186.4
ZMB 79379	adult	F	186.74
ZMB 79345	adult	M	187.66
ZMB 79365	adult	M	193.78
ZMB 79361	adult	F	197.27
ZMB 79363	adult	F	199.69
ZMB 79360	adult	M	202.24
ZMB 79366	adult	M	208.93
ZMB 79346	adult	F	213.64
ZMB 79378	adult	F	214.1
ZMB 79355	adult	F	217.2
ZMB 79377	adult	M	221.42
ZMB 79353	adult	M	222.62

Table 2. *Cynisca leucura* cranial shape variation. (A) Procrustes ANOVA results of cranial shape variation among juveniles and adults (age), males and females (sex), and their interaction (age×sex). (B) MANCOVA results of cranial shape allometry with size (logCS=log-transformed centroid size), and its variation with age (logCS×age), sex (logCS×sex), and their interaction (logCS×(age×sex)).

	df	SS	MS	R ²	F	Z	P
(A) Cranial shape variation							
Age	1	0.0245	0.0245	0.3882	23.477	12.275	0.0001
Sex	1	0.0009	0.0009	0.0175	0.5878	0.4971	0.7321
Age×sex	1	0.001	0.001	0.0188	0.9956	0.9989	0.3682
(B) Cranial shape allometry							
logCS	1	0.0285	0.0285	0.4523	30.553	14.051	0.0001
logCS×age	1	0.0017	0.0017	0.0267	1.8625	1.8068	0.0194
logCS×sex	1	0.0009	0.0009	0.0174	1.0477	1.0583	0.3214
logCS×(age×sex)	1	0.0011	0.0011	0.0208	1.2787	1.4536	0.0728

Figure 1. Taxon sampling. Head of *Cynisca leucura* (ZMB 79353) in (A) dorsal, (B) lateral and (C) ventral views, showing the distinct round-headed morphology. (D) *C. leucura* in life (photo courtesy of J. Penner) and (E) its habitat in northern Benin.
190x127mm (150 x 150 DPI)

Accept

Figure 2. Locations of 27 cranial landmarks used in geometric morphometric analyses.
81x98mm (300 x 300 DPI)

A

Figure 3. Cranial ontogeny of *Cynisca leucura*. (A) Ontogenetic allometry accounts for 45% of cranial shape variation during growth, with juveniles and adults exhibiting significantly different allometric slopes.

Symbols: Open=female, shaded=male, circle=adult, triangle=juvenile, X=juvenile of unknown sex. (B) Dorsal, lateral and ventral views of juvenile (left) and adult (right) specimens of *C. leucura*, illustrating small and large size extremes. Abbreviations: app, apical process of parietal; bo, basioccipital; ec, ectopterygoid; f, frontal; m, maxilla; n, nasal; ooc, otooccipital complex; os, orbitosphenoid; pal, palatine; p, parietal; pf, prefrontal; pm, premaxilla; pt, pterygoid; v, vomer. 150x142mm (300 x 300 DPI)

A

Figure 4. Major axes of cranial shape variation in *Cynisca leucura*. Warped cranial surfaces represent positive and negative shape extremes for the first two PC axes. Symbols: Open=female, shaded=male, circle=adult, triangle=juvenile, X=juvenile of unknown sex.
111x68mm (300 x 300 DPI)

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