ABSTRACT

Amphisabenia is a group of squamates adapted for a fossorial lifestyle. The skull is the animal's the main digging tool, and can present one of four principal shapes. The shovel-headed shape is considered to be the most specialized for digging. The South American genus *Leposternon* presents a shovel-headed morphotype, and is widely distributed on this continent. The general shovel-headed skull pattern may vary considerably, even within the same genus, and we hypothesized that this variation may be influenced primarily by body size and geographical factors. The present study investigated the variation in skull size and shape found among five *Leposternon* species, and examined the potential relationship between this variation and the size of the specimens and bioclimatic variables, through a geometric morphometric approach. Significant morphological variation was found among the species, and was also related systematically to body size and the geographical distribution of the specimens. As even subtle differences in the skull size or shape may represent significant modification in bite force and digging capacity and digging speed, the cranial variation found among the *Leposternon* species and specimens may have a direct influence on their diet and locomotor performance. Our results, together with direct observations of some of these species, suggest that shovel-headed amphisbaenians may be able to penetrate different soil types under a range of climatic conditions, especially considering the ample, but often sympatric distribution of the species studied here.

Keywords: allometry, Amphisbaenidae, biogeography, biomechanics, morphotype

1 INTRODUCTION

In the suborder Amphisbaenia, the skull is a compact, heavily ossified structure (Gans, 1969; Kearney, Maisano & Rowe, 2005) composed of four principal segments: 1) the anterior segment, known as the snout; 2) the intermediate segment of the braincase, which joins the snout to the occipital segment; 3) the posterior or occipital segment, and 4) the palatal series (Gans & Montero, 2009). The snout is the most variable of these segments, given its role as a digging tool (Gans & Montero, 2009). Amphisbaenians can be divided into four groups, based on the type of snout: shovel-headed, spade-headed, keel-headed, and round-headed (Kearney, 2003).

Amphisbaenian head morphotypes are related intimately with different patterns of excavating behavior, which range from random movements of the head (round-headed morphotype) and lateral movements (keel-headed) to shoveling (shovel-headed) and screwing (spade-headed) movements (Gans, 1974). The shovel-headed amphisbaenians include the genus *Leposternon*, and three other genera: *Rhineura*, *Dalophia*, and *Monopeltis* (Gans, 1974, 2005; Kearney, 2003), which have both the shovel-headed skull type and present “shoveling" movements of the head when excavating their galleries (Gans, 1974).

The excavatory cycle of *Leposternon microcephalum* and *Leposternon scutigerum* has been described as: 1) an initial static position with the gular and anterior body regions lying over the tunnel floor; 2) a retreating and downward bending of the head, with the tip of snout touching the floor substrate, and 3) a continuous upward and forward movement of the head, which compacts the granules of the substrate against the tunnel roof, while the pectoral region compresses the tunnel floor. This cycle ends with the animal dropping its head to return to its initial static position (Barros-Filho, Hohl & Rocha-Barbosa, 2008; Hohl et al., 2014; Hohl et al., 2017). The shovel-headed morphotype is considered to be the most specialized for digging, which allows these amphisbaenians to penetrate heavily compacted soils (Gans, 1974; Kearney, 2003; Gans 2005; Hohl et al., 2014).

The characteristics of the amphisbaenian head are also useful for phylogenetic studies (Kearney, 2003), including those that discuss about homoplasy and adaptive convergence related to fossoriality (Mott & Vieites, 2009; Gauthier et al., 2012), and provide valuable insights into the ecology of these animals through studies on their fossorial locomotion (e.g., Kaiser, 1955; Gans, 1960, 1974; Navas et al., 2004; Barros-Filho et al., 2008; Hohl et al., 2014; Hohl et al., 2017) and masticatory biomechanics (e.g., Baeckens et al., 2016). For example, small variations in the width of the posterior skull segment may have a relatively large impact on burrowing compression force and speed (Navas et al., 2004; Hohl et al., 2017), as well as bite force (Baeckens et al., 2016).

Studies have shown that the shape of the amphisbaenian skull may be influenced by a number of different factors. For example, the different head morphotypes (i.e., shovel, keel, round, and spade) may be related systematically to the geographic distribution of the different taxa (Gans, 1978; Kearney & Stuart, 2004; Mendes, 2017). In fact, the need to construct tunnel systems through the compression of the soil by movements of the head implies a close relationship between skull morphotypes and soil types (Gans, 1978). Mendes (2017) corroborated this assumption using a geometric morphometric approach, finding a strong correlation between the general shape of the skull and soil type, indicating that cranial diversity in the amphisbaenians is related systematically with burrowing habits. This author also alerted to the need for a more systematic analysis of amphisbaenian skull morphology, concluding that the suborder may encompass more variation than that found in the four principal morphotypes seen as standard in traditional studies.

In addition to being influenced by geographical factors, amphisbaenian skull shape may also vary in relation to body size. Gans & Montero (2009), for example, demonstrated that the large species of the (round-headed) genus *Amphisbaena* have skulls of a different shape in comparison with the smaller species. Magwene (1997) and Hipsley et al. (2016) also demonstrated, respectively, significant changes in the shape of the skull of *Amphisbaena caeca* and *Cynisca leucura* during their ontogenetic development.

The genus *Leposternon* currently has 11 species, including the new species, *Leposternon mineiro* Ribeiro, Silveira & Santos-Jr, 2018, from the Brazilian Cerrado biome. Uetz & Hosek (2018) proposed that the species can be distinguished primarily by the morphology of the cephalic shields, the number of annuli in the body and tail, the number of vertebrae, and the presence or absence of precloacal pores (Gans, 1971; Porto, Soares & Caramaschi, 2000; Ribeiro, Vaz-Silva & Santos-Jr, 2008). The osteological features of the skull have also been used to differentiate *Leposternon* species (see Barros-Filho, 2000).

The genus *Leposternon* is distributed in South America, between the Brazilian Amazon basin and the Pampas of northern Argentina (Perez & Ribeiro, 2008; Ribeiro et al., 2008; Ribeiro et al., 2011; Ribeiro, Santos-Jr & Zaher, 2015). Some of *Leposternon* species are widely distributed within this area, whereas others have a more restricted range. Species with more ample ranges are subject to more diverse climatic conditions and soil types, and consequently, to different adaptive pressures.

The general shovel-headed skull pattern may vary even within the same genus, such as *Leposternon*, and we hypothesize that these differences may be influenced primarily by body size and geographical factors. In this context, the present study investigated the variation in skull size and shape found among five *Leposternon* species, and examined the potential relationship between this variation and the size of the specimens and bioclimatic variables, such as temperature and precipitation, through a geometric morphometric approach. The soil types that the specimens likely encounter at the respective collecting localities were also identified.

2 MATERIAL AND METHODS

2.1 Sample studied

Amphisbaenians are often poorly represented in museum collections due to their fossorial lifestyle (Navega-Gonçalves, 2004; Barros-Filho et al., 2008; Mendes, 2017). In the present study, we analyzed 61 well-preserved skulls of adult specimens belonging to five of the 11 *Leposternon* species known to occur in South America. These species were *Leposternon scutigerum* (Hemprich, 1820), *Leposternon microcephalum* Wagler, 1824, *Leposternon polystegum* (Duméril, 1851), *Leposternon infraorbitale* Berthold, 1859, and *Leposternon wuchereri* (Peters, 1879). While adult body size may vary considerably in this genus (Barros-Filho, 2000), we considered specimens with a body length of more than 150 mm to be adults (Gans, 1971). We recorded the geographic coordinates of the collecting locality of each specimen, resulting in a list of 23 localities in eight Brazilian states (Bahia, Ceará, Goiás, Pará, Paraná, Rio de Janeiro, Rondônia, and Santa Catarina), as well as Paraguay and Argentina (Figure 1).

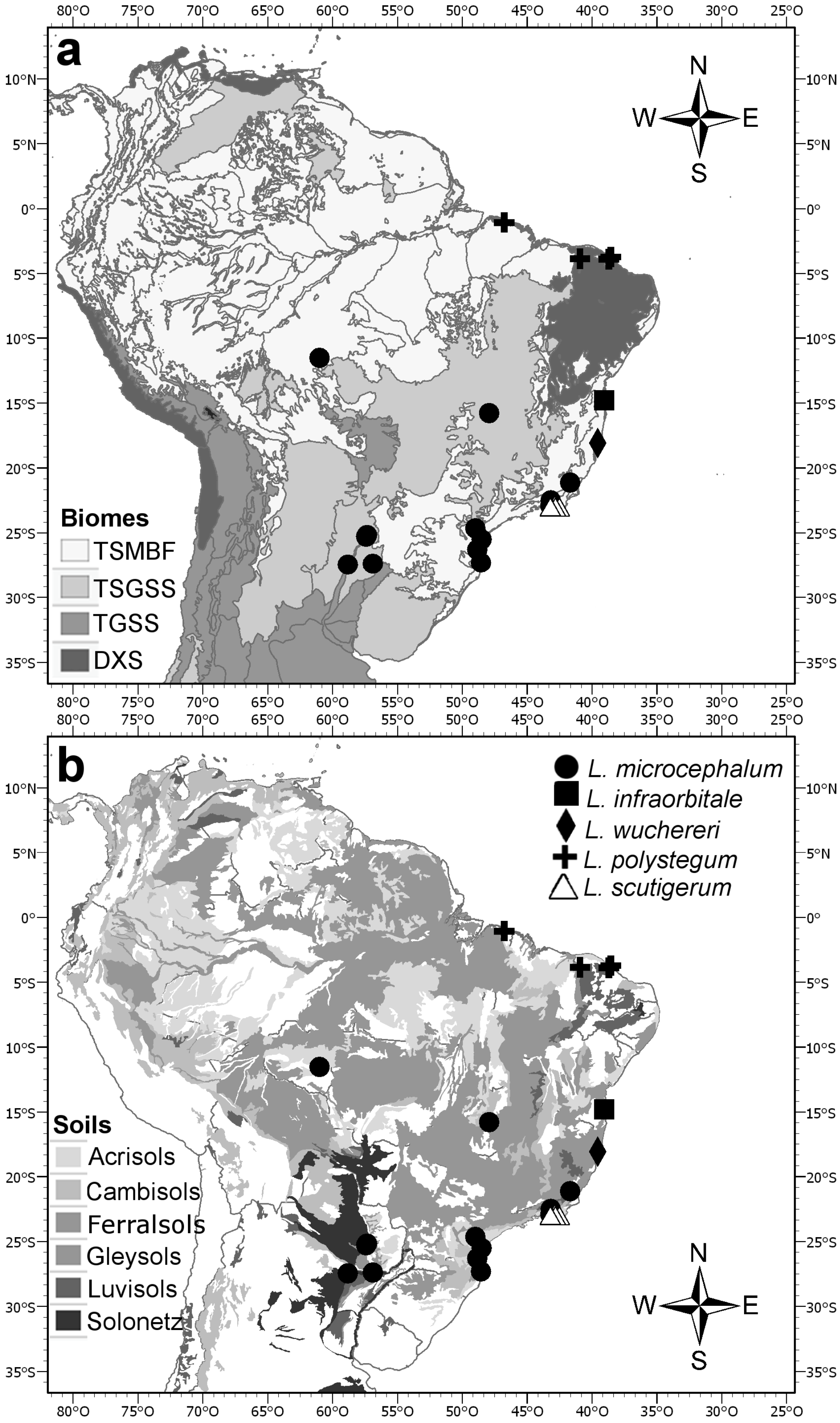

Figure 1.— Distribution of the samples analyzed in the present study in the different South American biomes (A) and soil types (B). The symbols indicate approximate

locations of the specimen collecting sites, distinguished by species, and may refer to more than one specimen. TSMBF = Tropical and Subtropical Moist Broadleaf Forest; TSGSS = Tropical and Subtropical Grassland, Savanna and Scrubland; TGSS = Temperate Grassland, Savanna and Scrubland; DXS = Deserts and Xeric Scrubland.

Some *Leposternon* species have a relatively ample distribution. *Leposternon wuchereri*, for example, is found in the Caatinga and Atlantic Forest biomes in the Brazilian states of Bahia, Espírito Santo, and Rio de Janeiro, while *L. infraorbitale* occurs in the Cerrado, Caatinga, and Atlantic Forest biomes in the states of Mato Grosso, Goiás, Pernambuco, Bahia, Espírito Santo, Minas Gerais, and Rio de Janeiro. *Leposternon polystegum* occurs in the Amazon, Atlantic Forest, Caatinga, and Cerrado biomes, in the states of Pará, Rio Grande do Norte, Pernambuco, Bahia, Ceará, and Maranhão (Gans, 1971; Rocha et al., 2009), and *L. microcephalum* has the widest distribution of the study species, being found in all Brazilian biomes, as well as neighboring areas of Bolivia, Argentine, Paraguay, and Uruguay (Perez & Ribeiro, 2008; Ribeiro et al., 2011).

The fifth species studied here, *L. scutigerum*, has a much more restricted distribution, and is in fact endemic to the Brazilian state of Rio de Janeiro (Barros-Filho, 1994; Rocha et al., 2009). This species is listed as Endangered (EN) by the IUCN (Colli et al., 2016), and is also on the Brazilian Red List of endangered species (see federal ordinance MMA no. 444 of December 17th, 2014).

Leposternon microcephalum was collected at the largest number of localities (13), followed by *L. polystegum* with four localities, *L. infraorbitale* (two localities) and *L. wuchereri* (one locality). Given its endemism, *L.scutigerum* was sampled in three municipalities in the state of Rio de Janeiro (Barros-Filho, 1994; Rocha et al., 2009). The species are distributed between females, males, and unknown as follows: *L. infraorbitale* (5:0:1), *L. polystegum* (5:8:2), *L. wuchereri* (4:2:4), *L. scutigerum* (1:3:2), and *L. microcephalum* (5:14:5). A full list of the specimens, their sex, collecting locality, and the museum in which the specimen is deposited is given in Appendix I.

2.2 Geometric Morphometric procedures

A two-dimensional Geometric Morphometric analysis, based on landmarks and semi-landmarks (Bookstein, 1991, 1997; MacLeod, 2013), was used to evaluate the variation in skull characteristics among the five *Leposternon* species, and provided preliminary insights into the influence of body size and geographic factors on this variation. The skulls were photographed in dorsal and lateral views at a standard distance (3.5 cm) on a photographic table with a Sony DSC-HX1digital camera. The specimen and camera were fixed in place, and adjusted with a builder's level to ensure that the plane of the camera and the axis of the maximum skull length were exactly parallel to one another. Given the small size of the structures being registered, the camera's macro function was enabled to ensure the resolution of the details. A scale bar was placed in each shot, and the skulls were placed in a standard position during all shots (Figure 2).

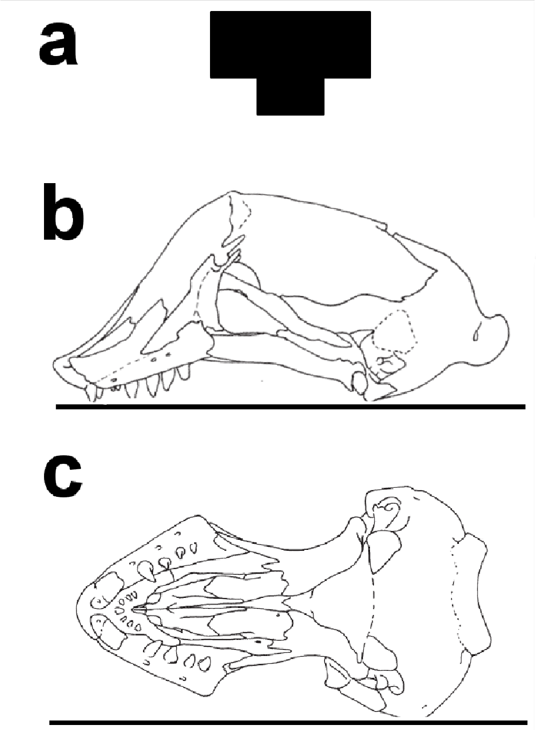

Figure 2.— Position of the camera (A) in relation to the skulls when photographed its dorsal (B) and lateral (C) views. The diagrams of the *Leposternon microcephalum* specimen were obtained from Gans & Montero (2009).

We prioritized type I and II landmarks to describe the skull traits (Bookstein, 1991). Curved structures, such as the contours of the snout, and points that were difficult to mark (e.g., the lateral limits of the intermediate segment) were described using semi-landmarks. Following MacLeod (2013), landmarks and semi-landmarks were analyzed altogether without any *a priori* mathematical adjustment (i.e., "sliding of the sami-landmarks"). Ten landmarks and six semi-landmarks were used in the dorsal analysis of the skulls (Figure 3A), and nine landmarks and five semi-landmarks were used in the lateral analysis (Figure 3B). We used the MakeFan6 software (Sheets, 2002) to create the "combs" used to plot the semi-landmarks.

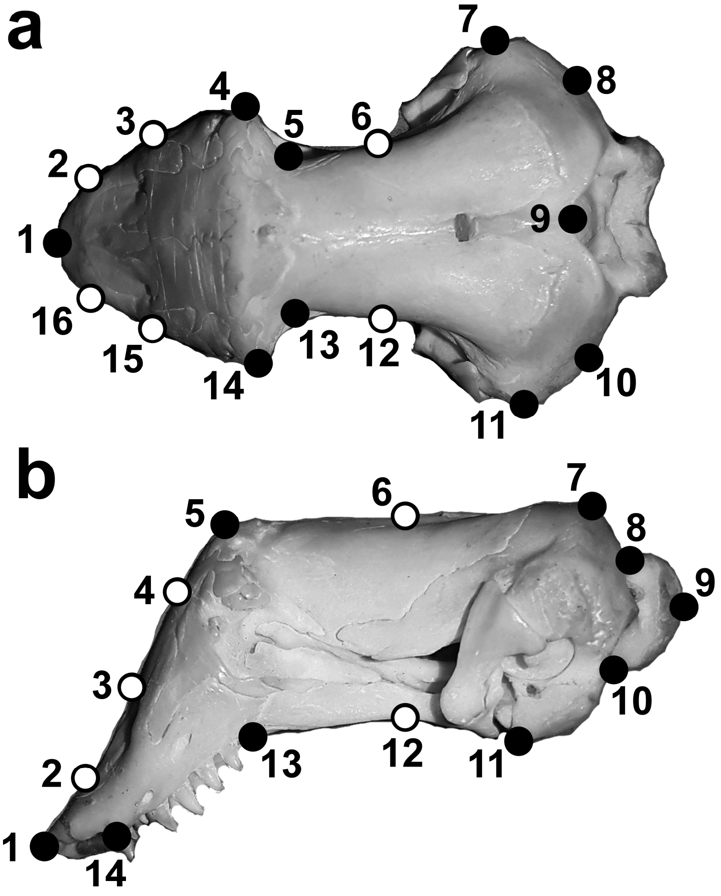

Figure 3.— Position of the landmarks (black circles) and semi-landmarks (white circles) on the skull of a *Leposternon microcephalum* specimen (UnB 3663) used to describe the dorsal (A) and lateral (B) views.

In the dorsal skull view, landmarks 1 and 9 were used as reference points for the "comb", while in the lateral view, the pairs of landmarks 1 and 5, and 5 and 7 were used for this purpose. The landmarks and semi-landmarks were acquired using TPSDig2, version 2.0 (Rohlf, 2004). Each landmark was digitized twice to estimate measurement error. This repetition was conducted two weeks after the first round of data collection (see Viscosi & Cardini, 2011), but no statistically significant difference was found. The anatomical nomenclature of the *Leposternon* skull bones followed Gans & Montero (2009). A full description of the landmarks and semi-landmarks is presented in Appendix II.

The raw coordinates (*x*, *y*) were superimposed in a Procrustes procedure to analyze size and shape separately and minimize the differences in size, translation, and rotation of the specimens (Rohlf & Slice, 1990). The size of each specimen is given as the Centroid Size (CS) of the landmark configuration (Viscosi & Cardini, 2011). After obtaining the Procrustes coordinates in MorphoJ (Klingenberg, 2011), we used TPSSmall v.1.34 to test whether the variation in shape among the specimens was within the required limits, to permit analytical procedures based on the tangent space approximation, such as the thin-plate-spline methods (Rohlf, 2017). The software regresses through the origin of the set of Euclidean distances in Euclidean space onto the set of Procrustes shape distances. If optimal, this approximation will return a regression with both slope and correlation virtually equal to 1 (Viscosi & Cardini, 2011). The samples were also inspected for outliers in MorphoJ.

2.3 Interspecific analysis

The variation in the size of the different species was tested using a univariate ANOVA of the log-transformed Centroid Size (logCS) values (Appendix III). We opted to log-transform the values because of the considerable variation in size found among the adult specimens (see Klingenberg, 2011). Given this variation (Barros-Filho, 2000), failure to recognize allometric change may result in overweighting in the phylogenetic analysis (Gans & Montero, 2009). To verify the real variation in shape among the species analyzed, we first regressed shape (i.e., the Procrustes coordinates) on size (logCS). The results of this regression were used to characterize the variation in shape related to body size. This allometric bias determined the use of the residuals of the regressions in all subsequent analyses (e.g., CVA) as a means of correcting for the influence of body size on the shape data (Klingenberg, 2011).

A Principal Components Analysis (PCA) was used to ordinate the specimens in morphological space (Monteiro & Reis, 1999; Zelditch et al., 2004) and identify the vector of maximum variance within the shape data. As the PCA is not considered to be a conclusive statistical test (Webster & Sheets, 2010), the statistical significance of the morphological differences between species was assessed using a Canonical Variate Analysis (CVA) of the residuals of the regression of shape on size. All geometric morphometric procedures were run in MorphoJ. Shape changes were described by observing the major variation axes and were represented by wireframes (Viscosi & Cardini, 2011).

2.4 Biogeographical analysis

The ample distribution of the genus *Leposternon* in South America (Ribeiro et al., 2015) led us to investigate whether skull size and shape vary systematically by latitude and longitude, given that both these parameters are considered to be commonly correlated with climatic variation (see Corti et al., 1996; Barčiová, Šumbera & Burda, 2009; Cáceres et al., 2013; Meloro et al., 2014). To better understanding the influence of climate, 19 standard bioclimatic variables were tested directly. These variables were downloaded with a resolution of 10 arc-minutes (~340 Km^2) from WorldClim, version 2 (Fick & Hijmans, 2017), after being extracted using ArcGIS Pro 2.1.3 (Esri, 2018). As this study involves fossorial animals, the main soil type of each specimen collecting locality was obtained from the Harmonized World Soil Dataset – Major Soil Groups (FAO/IIASA/ISRIC/ISS-CAS/JRC, 2009), extracted using ArcGIS Pro 2.1.3. Specimens that lacked information on the collecting locality (*L. microcephalum* ZUFRJ 1514, 1282, 975, and *L. wuchereri* ZUFRJ 1555) were excluded from these analyses.

Regressions using logCS as the dependent variable and latitude and longitude, separately, as independent variables were run to verify size variation over the geographical distribution of the species. This analysis was repeated using the Procrustes coordinates as the dependent variables to verify the influence of latitude and longitude on shape variation.

A two-block Partial Last Squares (PLS) analysis (Rohlf & Corti, 2000) was run in MorphoJ to test for a possible relationship between environment and skull shape.

The shape variables (Procrustes coordinates) constituted Block 1 and the 19 bioclimatic variables, Block 2, following Meloro et al. (2014). Both blocks are weighted equally in PLS models. The PLS extracts vectors from the correlation matrix of each block so that the degree of co-variation between blocks is maximized. While this study is the first of its kind on amphisbaenians, this technique is now standard in geometric morphometric applications that test the association between skull shape and environmental variables in mammals (cf. Cáceres et al., 2013; Meloro et al., 2014; Bubadué et al., 2016). Prior to this analysis, the environmental variables were log-transformed to standardize the values (Klingenberg, 2011). The PLS1 Shape scores were then analyzed using the Kruskal-Wallis nonparametric analysis of variance, followed by the Mann-Whitney pairwise test to verify differences between the *Leposternon* species along the environmental gradient, and the PLS1 Climate scores were regressed on latitude and longitude to verify the possible correlations between these variables (see Cáceres et al., 2013).

3 RESULTS

For the analyses of both skull views, the slope of the regression of the Euclidean distances in the tangent space on the Procrustes shape distances in the curved Procrustes shape space was 0.999, with a correlation of 1.000. The approximation was therefore assumed to be optimal for the skull views analyzed.

3.1 Interspecific analysis

The one-way ANOVA found significant differences in size among *Leposternon* species in both the dorsal and lateral views of the skull (Dorsal - $F_{4, 52} = 22.75$, $P < 0.001$; Lateral - $F_{4, 57} = 19.57$, $P < 0.0001$). Tukey's post-hoc test revealed significant differences between *L. infraorbitale* and all other species in both skull views, with a significant size difference also being found between *L. microcephalum* and *L. wuchereri*, but in the dorsal view alone (Table 1). *Leposternon infraorbitale* was the species with the largest skull (Figure 4).

TABLE 1 Results of Tukey's *posthoc* test for the size differences between the pairs of the *Leposternon* species analyzed in the present study(dorsal and lateral views of the skull), showing the Q values (lower diagonal) and the *P* values (upper diagonal). Significant values are highlighted in bold type.

Dorsal view	*L. infraorbitale*	*L. polystegum*	*L. wuchereri*	*L. scutigerum*	*L. microcephalum*
L. infraorbitale	—	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
L. polystegum	12.47	—	0.7864	0.5559	0.399
L. wuchereri	14.08	1.608	—	0.0752	**< 0.05**
L. scutigerum	10.32	2.147	3.754	—	0.9991
L. microcephalum	9.957	2.51	4.118	0.3634	—
Lateral view					
L. infraorbitale	—	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
L. polystegum	12.39	—	0.8988	0.4333	0.5836
L. wuchereri	13.65	1.261	—	0.0835	0.141
L. scutigerum	9.97	2.427	3.688	—	0.9993
L. microcephalum	10.31	2.085	3.346	0.3422	—

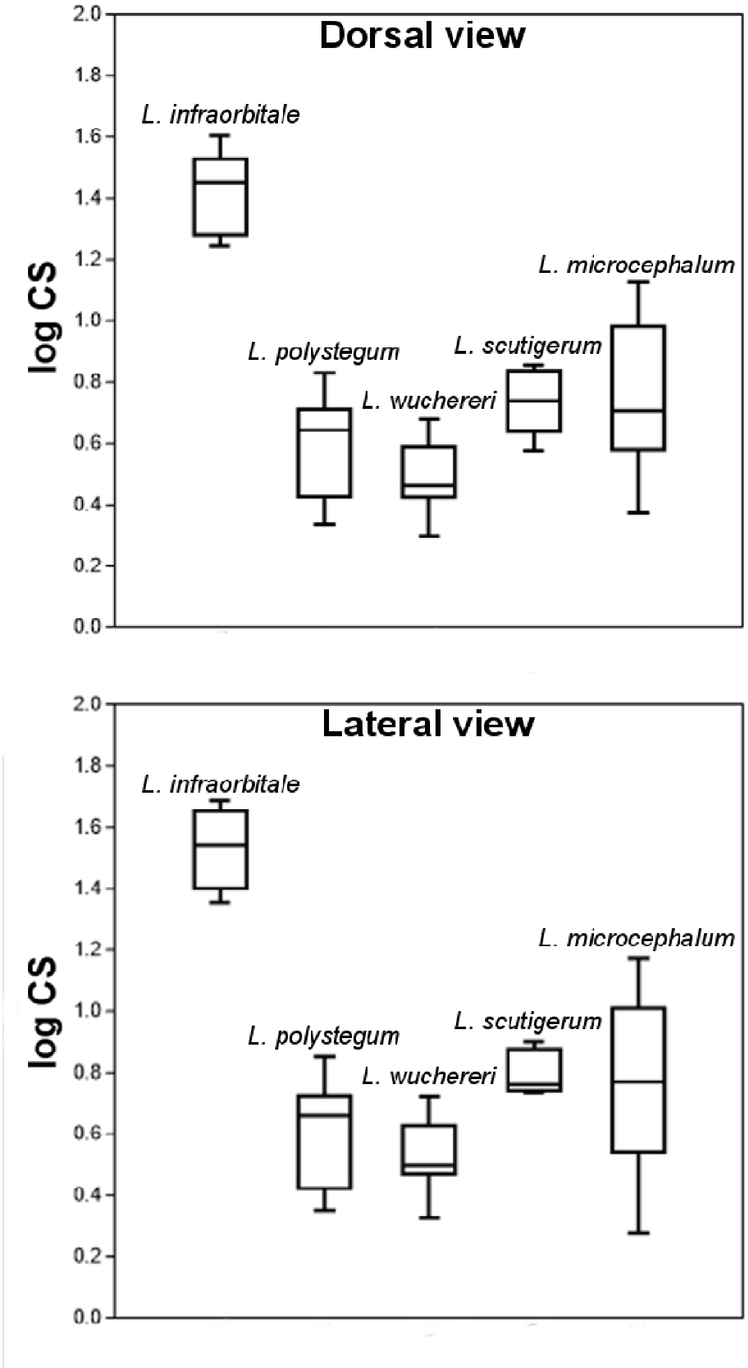

Figure 4.— Box plot of the log-transformed Centroid Sizes for the dorsal and lateral skull views in five species of *Leposternon*. Horizontal line = median; box = first interquartile; Vertical bar = second interquartile.

The regression of the Procrustes coordinates on logCS indicated a significant influence of body size on the shape of the skull in both views (dorsal - $P < 0.0001$ and lateral - $P = 0.0014$). However, a high percentage of prediction was obtained only for the dorsal view (Total Sums of Squares = 0.199; Predicted = 29.8%) compared with the lateral view (Predicted = 5.8%). We thus described the allometric variation in shape only for the dorsal view of the skull.

The wireframes demonstrated that smaller specimens (logCS close to 0.3) presented a relatively larger and more rounded snout, with a more shovel-like shape, a markedly thicker intermediate segment, and posterior segment less laterally projected, at the optical capsules, with its posterior contour rounded (Figure 5A). By contrast, the larger specimens (logCS nearing 1.6) had a discrete lateral compression of the anterior segment, also presenting a shovel shape in this area, a constricted intermediate segment, and a more laterally projected posterior segment, with its posterior border almost straight (Figure 5A).

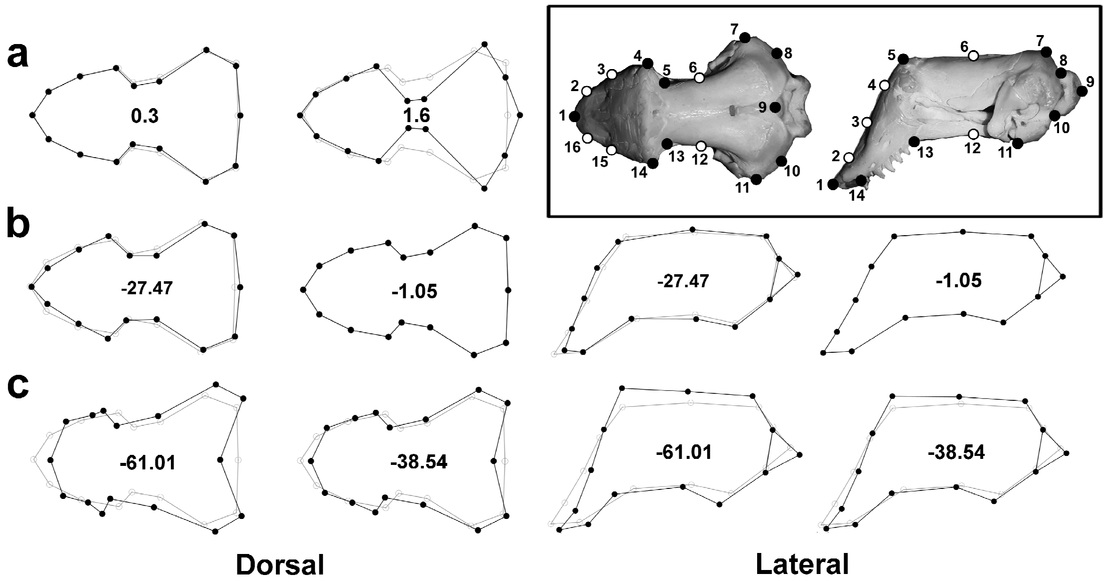

Figure 5.— Wireframes of the deformation related to the minimum and maximum values of the log-transformed Centroid Size (A), according to latitude (B) and longitude (C). Note that differences in skull shape related to the size of the specimen are not shown for the lateral view of the skull. The box contains the numbers and positions of the landmarks and semi-landmarks. The light grey in the wireframes represent the mean shape for the data set.

In the PCA of the dorsal view of the skull, the first axis (PC1) explained 37.24% of the variation in the specimens, and the second axis, 21.73% (total of 58.97%). *Leposternon microcephalum* presented the widest range along the PC axes, returns mostly positive PC1 scores and negative PC2 scores. *Leposternon* *infraorbitale* was well distinguished from the other species, with the most positive scores on PC1. *Leposternon scutigerum* and *L. microcephalum* are well distinguished along PC2, with positive and negative scores, respectively. *Leposternon polystegum* and *L. wuchereri* presented negative scores on PC1, but, while they were well separated from the other species, they were superimposed (Figure 6).

The skull variation found along PC1 showed that specimens with positive scores (e.g., *L. infraorbitale* and most *L. microcephalum* specimens) tend to have a minimized, triangular-shaped snout area, associated with a narrow intermediate segment, and a robust occipital region with an almost straight posterior border. By contrast, the specimens with negative scores (e.g., *L. wuchereri*, *L. polystegum*, and *L. scutigerum*) tend to have a rounder and more expanded snout area with a robust intermediate segment, and a discretely slender occipital portion with its posterior border rounded (Figure 6).

On the PC2 axis, specimens with the most positive scores (e.g., *L. scutigerum* and *L. infraorbitale*) tend to have a laterally-expanded anterior segment associated with an elongation of the anteriormost point of the snout, forming a shovel-like shape, with a narrow intermediate segment, and a robust occipital segment. In addition, the lateral limits of the anterior and posterior segments are closely parallel. Specimens in the extreme negative range of PC2 scores (e.g., almost all *L. microcephalum* specimens) presented a shortened and narrowed snout, and thicker intermediate and occipital portions. The lateral limits of the anterior and posterior segments are separated from one another (Figure 6).

The PCA for the lateral view of the skull presented considerable overlap of the species, except for *L. polystegum*, which was relatively distinct. Here, the first axis (PC1) explained only 26.98% of the variation in the specimens, and the second axis, 19.24% (total of 46.22%). *Leposternon microcephalum* presented the widest range of PC scores, with individuals distributed along the positive and negative ranges of both axes. *Leposternon polystegum* was well distinguished from the others species, due to having the most negative PC1 scores (Figure 6).

Skull variation along the PC1 indicated that specimens with more positive scores (e.g., some *L. microcephalum* and *L. infraorbitale* specimens, and *L. wuchereri*) tend to have a retracted anterior segment, with the snout tip shortened, pointing downward, and a convex anterior border, and dorsoventrally robust intermediate and posterior segments. In the specimens with the most negative scores (*L. polystegum*), on the other hand, the anterior segment of the skull is projected anteriorly, and elongated, with a concave anterior border, and the intermediate and posterior segments are dorsoventrally narrow (Figure 6).

Specimens with the most positive scores on the PC2 axis (e.g., some *L. microcephalum* specimens, *L. infraorbitale* and *L. wuchereri*) tend to have the snout projected forward and shortened, with a concave anterior border, and the intermediate and posterior segments narrowed dorsoventrally. Specimens at the negative extreme of PC2 (e.g., some *L. microcephalum*) presented an anterior segment projected ventrally and elongated, with the anterior border almost straight, and the intermediate and posterior segments thicker dorsoventrally (Figure 6).

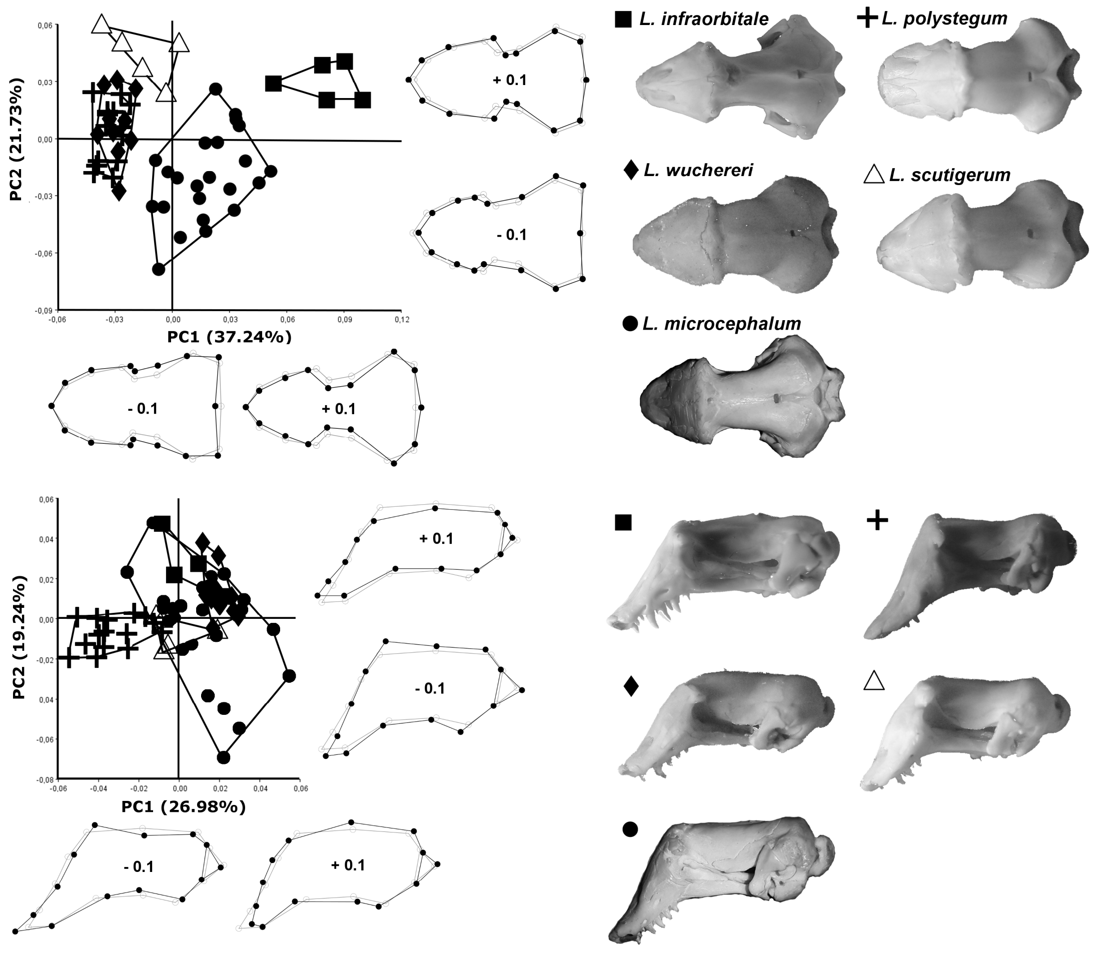

Figure 6.— Scatterplots of the first pair of Principal Components (PC1 vs. PC2) for the dorsal and lateral skull views, showing the variation in the shape of the species through the wireframes, relative to the negative and positive extremes of the PC

axes. At right, photographs of specimens selected randomly to represent the five species analyzed in both skull views. The light grey in the wireframes represent the mean shape for the data set.

In both skull views, the *P* values of the CVA permutation tests (10,000 permutation) on the residual values of the regression (corrected for body size), revealed significant differences among the five *Leposternon* species analyzed in the present study (Table 2).

TABLE 2 Results of the CVA for the morphometric variation found between the pairs of the *Leposternon* species analyzed in the present study (dorsal and lateral views of the skull), showing the Procrustes distances (lower diagonal) and *P* values (upper diagonal).

Dorsal view	*L. infraorbitale*	*L. microcephalum*	*L. polystegum*	*L.scutigerum*	*L. wuchereri*
L. infraorbitale	—	**< 0.0001**	**< 0.0001**	**0.0080**	**0.0002**
L. microcephalum	0.0501	—	**< 0.0001**	**< 0.0001**	**< 0.0001**
L. polystegum	0.0536	0.0558	—	**< 0.0001**	**< 0.0001**
L.scutigerum	0.0728	0.0760	0.0630	—	**0.0006**
L. wuchereri	0.0431	0.0489	0.0524	0.0418	—
Lateral view					
L. infraorbitale	—	**0.0133**	**0.0003**	**0.0065**	**0.0033**
L. microcephalum	0.0439	—	**< 0.0001**	**<0.0318**	**0.0095**
L. polystegum	0.0606	0.0501	—	**0.0001**	**< 0.0001**
L.scutigerum	0.05	0.0364	0.0427	—	**0.0008**

L. wuchereri	0.0371	0.0291	0.0583	0.0383	—

3.2 Biogeographical analysis

The analyses indicated that skull size did not vary with latitude or longitude, although both these parameters are related significantly to the variation in skull shape found among the *Leposternon* specimens (Table 3). Latitude explained a higher percentage of shape variance (Dorsal skull view - Predicted: 13.22%; Lateral skull view – 17.42%) compared with longitude (Dorsal view - 10.1%; Lateral view – 6.96%).

TABLE 3 Regression models of the size (logCS) and shape variables (Procrustes coordinates) as the dependent variables and the latitude and longitude (in decimals) as the independent variables, based on the South American *Leposternon* dataset compiled in the present study. Significant *P* values are highlighted in bold type. The *N* value is the number of localities. TSS = Total Sum of Squares. Predicted % is the percentage of covariance explained by the analysis.

Dorsal view (*N* = 22)	Predictors	TSS	Predicted %	*P*
logCS	Lat	4.55	1.54	0.381
	Long	4.55	3.3	0.196
Procrustes coord.	Lat	0.18	13.22	**< 0.0001**
	Long	0.18	10.1	**0.0002**
Lateral view (*N* = 23)				
logCS	Lat	4.98	1.72	0.354
	Long	4.98	4.95	0.109
Procrustes coord.	Lat	0.12	17.42	**< 0.0001**
	Long	0.12	6.96	**0.0009**

Overall, the analysis of the variation in the shape of the *Leposternon* skull, based on the dorsal view, indicates that specimens at higher latitudes (e.g., 27.47 S) tend to have a more pointed anterior snout extremity, with a large transversal crest, forming the triangular shape of the anterior segment, with intermediate segment more narrowed, and the posterior segment more robust (Figure 5B). Specimens collected near the equator (e.g., 1.05 S) had a larger and more rounded snout, with a more shovel-like shape, with a discretely thicker intermediate segment and more robust posterior segment (Figure 5B).

Considering the variation in skull morphology among longitudes, the specimens collected at localities located more to the west (e.g., 61.01 W) had a shorter snout, thickened laterally with a pronounced transversal crest, and robust intermediate and posterior segments, while the displacement of landmark 9 indicates a relatively large foramen magnum opening (Figure 5C). By contrast, while specimens from lower longitudes (e.g., 38.54 W) had almost the same skull configuration, the snout was longer with a less pronounced transversal crest, and the posterior segment was discretely thinner, with a narrower foramen magnum (Figure 5C).

In the lateral view, the main differences in skull morphology were observed in the snout segment. Specimens from higher latitudes (e.g., 27.47 S) tend to have an indented anterior segment with the tip of the snout shortened and pointing downward, with a convex anterior border, and the intermediate and posterior segments discretely thicker dorsoventrally (Figure 5B). In the specimens collected near the equator (e.g., 1.05 S), the anterior segment of the skull is projected in the anterior direction and elongated with a discretely concave anterior border, with intermediate and posterior segments less robust dorsoventrally (Figure 5B).

Longitudinally, the main differences were observed in the intermediate and posterior segments. Western specimens (e.g., 61.01 W) had an indented snout segment, with more robust intermediate and posterior segments (Figure 5C). Specimens from lower longitudes (e.g., 38.54 W) had almost the same skull configuration, but the snout was less indented, and the intermediate and posterior segments were discretely thinner (Figure 5C).

In the dorsal view of the skull, PLS analysis produced 19 pairs of vectors (PLS scores between blocks). The PLS1 explained 91.38% of the covariation between bioclimatic variables and shape, and PLS2, 6.7% (total of 98.08%). These two principal pairs of vectors of covariation were strong and statistically significant (PLS1 – $R = 0.75$, $P < 0.0001$; PLS2 – $R = 0.6$, $P < 0.001$).

A Kruskal-Wallis test indicated significant differences among species in the PLS1 Shape scores ($N = 53$; H = 45.21; $P < 0.0001$), and the Mann-Whitney comparisons found significant differences between all pairs of species, except between *L. scutigerum* and *L. wuchereri* ($P = 0.286$). The scatterplot of the PLS1 of the shape block (PLS1 Shape) versus the PLS1 of the block of climate variables (PLS1 Climate) thus supports the separation of the species along an environmental gradient (Figure 7). The PLS1 Climate was related significantly to latitude (TSS: 32.83, Predicted: 83.54%, $P < 0.0001$).

The PLS1 Climate was loaded negatively on Temperature Seasonality, Annual Temperature Range, and the precipitation of the Driest Month, the Driest Quarter, and the Warmest Quarter. The most positive correlations of PLS1 Climate scores were found with Precipitation Seasonality, and the precipitation of the Wettest Month, the Wettest Quarter and the Coldest Quarter. In practice, the PLS1 Climate analysis discriminated between localities that have rainy winters and those that have rainy summers.

Specimens from localities with rainy summers tend to have skulls with a more pointed anterior segment, and a large transversal crest, conferring a triangular shape on the segment, with a narrowed intermediate segment, and a robust posterior segment (*L. infraorbitale* and most *L. microcephalum*). Specimens from localities with rainy winters tend to have a large and more rounded snout, with a more shovel-like shape, a discretely thicker intermediate segment, and robust posterior segment (*L. polystegum*). *Leposternon scutigerum* and *L. wuchereri* appeared to present an intermediate shape between these two extremes under the same climatic conditions faced by *L. infraorbitale* and most *L. microcephalum*.

The PLS analysis also produced 19 pairs of vectors for the lateral view of the skull (Figure 7). The PLS1 explained 94.6% of the covariation between the bioclimatic variables and skull shape, and the PLS2, 4.4% (total of 99%). The first vector was strong and statistically significant (PLS1 – $R = 0.88$, $P < 0.0001$), and the second was also significant (PLS2 – $R = 0.52$, $P = 0.004$). The Kruskal-Wallis analysis found significant variation among the species in the PLS1 Shape scores (*N*

= 53; H = 39.39; *P*< 0.0001), with the Mann-Whitney test indicating significant differences between all pairs of species, except between *L. infraorbitale* and *L. wuchereri* (*P* = 0.487) and *L. microcephalum* (*P* = 0.727). The PLS1 Climate was significantly related to latitude (TSS: 32.24, Predicted: 86.37%, *P* < 0.0001).

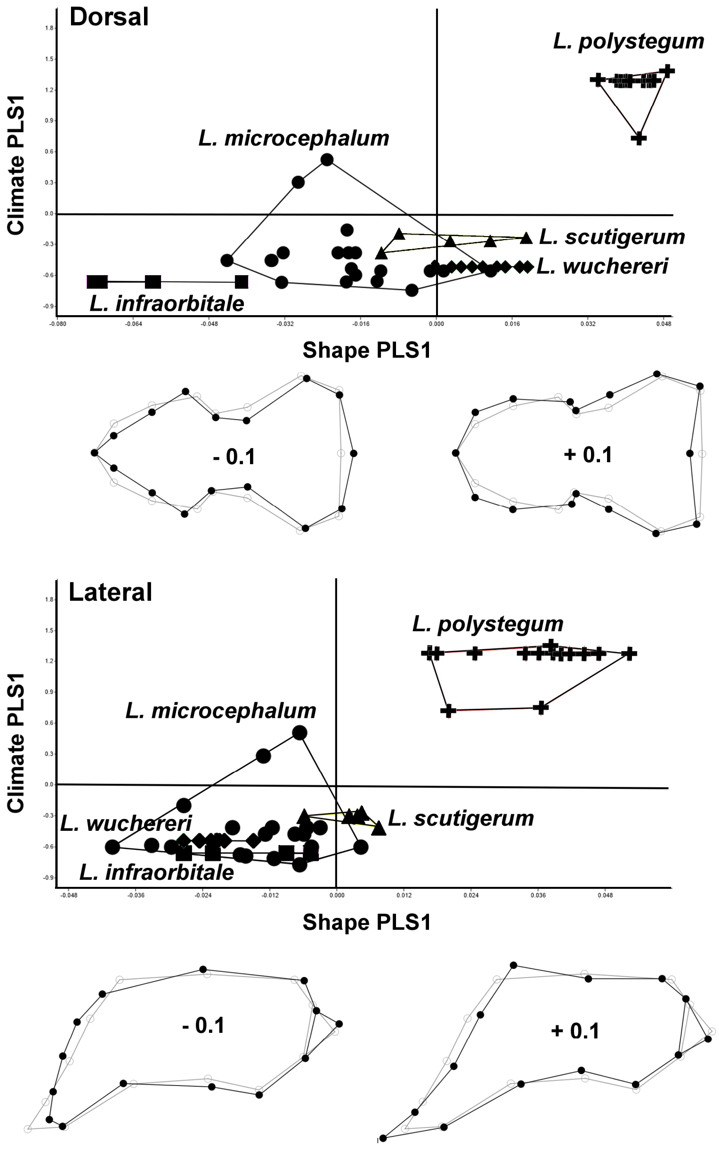

Figure 7.— Scatterplot of the PLS1 Climate vs. PLS1 Shape associated with the wireframes, from the most negative to the most positive PLS scores. The light grey in the wireframes represent the mean shape for the data set.

The PLS1 Climate was loaded negatively on Temperature Seasonality, Annual Temperature Range, and the precipitation of the Driest Month, the Driest Quarter, and the Warmest Quarter. The most positive correlation of the PLS1 climate scores were recorded with Precipitation Seasonality, and the precipitation of the Wettest Month, the Wettest Quarter, and the Coldest Quarter.

The skulls of specimens collected at localities with rainy summers tend to have an indented anterior segment, with the tip of the snout shortened and pointing downward, and a convex anterior border, with intermediate and posterior segments thickened discretely dorsoventrally (seen in some *L. microcephalum* specimens, *L. wuchereri* and *L. infraorbitale*). In the specimens collected from localities with rainy winters, the anterior segment of the skull tends to be projected anteriorly, and elongated with a discretely concave anterior border, and intermediate and posterior segments less robust dorsoventrally (*L. polystegum*). *Leposternon scutigerum* appears to present an intermediate shape between these two extremes, under the same climatic conditions as those faced by *L. infraorbitale*, *L. microcephalum*, and *L. wuchereri*.

Based on the data from the Harmonized World Soil Dataset and the geographic coordinates of the collecting localities, the *Leposternon* specimens analyzed in the present study occurred in six soil types (Acrisols, Cambisols, Ferralsols, Gleysols, Luvisols, and Solonetz). *Leposternon polystegum* occurred in Acrisols and Ferralsols, *L. wuchereri* and *L. infraorbitale* in Ferralsols, *L. scutigerum* in Acrisols, and *L. microcephalum* in all six soil types.

4 DISCUSSION

4.1 Size influencing shape

Similar allometric variation was found in the species of the round-headed genus *Amphisbaena* (Gans & Montero, 2009), in which the larger species, such as *Amphisbaena alba, Amphisbaena bolivica,* and *Amphisbaena angustifrons*, presented an hourglass-shaped skull in the dorsal view, with a marked constriction posterior to the maxilla (i.e., covering the intermediate segment) and a narrowed anterior segment (i.e., snout), while in the smaller species, such as *Amphisbaena lumbricalis* and *Amphisbaena slevini*, the skull is as tube-shaped with no constriction posterior to the maxilla, but with a rounded snout. Allometric variation was also found in all the *Leposternon* species analyzed here, with a more pronounced pattern being observed in the species represented by larger samples (*L. polystegum*, *L. wuchereri*, and *L. microcephalum*).

As the intermediate segment of the skull is the origin of most of the animal's adductor muscles (Gans & Montero, 2009), the marked constriction observed in this segment in the larger *Leposternon* specimens implies an increase in muscle mass, which may be related to a shift in diet or an increase in the force of the bite, as suggested by Magwene (1997) in a study of the ontogeny of *A. caeca*. The robustness of the posterior region is also related to bite force, and Vanhooydonck et al. (2011) and Baeckens et al. (2016) concluded that bite performance increases with head width. Our results indicated that this segment is more projected laterally in larger specimens. In the adults, then, larger individuals would be able to access more types of prey.

The solid occipital segment is also the insertion site of the nuchal muscles that moves the entire head (Gans & Montero, 2009). The *longissimus dorsi*, for example, is an extensive dorsal muscle that originates at the base of the head (proximal portion) and runs backward along the dorsal region of the animal, forming the distal portion (Navas et al., 2004). In the shovel-headed amphisbaenians, specifically *L. microcephalum*, even small variations in head width may have a major impact on burrowing compression force (Navas et al., 2004) and speed (Hohl et al., 2017). In this case, the analyses of the morphological configuration of the skull presented here indicate that the larger *Leposternon* specimens or species are able to exert a higher compression force, but dig more slowly, reflecting a trade-off between compression forces and burrowing speeds, as suggested by Hohl et al. (2017).

4.2 Interspecific skull variation

Barros-Filho (2000) described the interspecific variation in the skull of *Leposternon*, including the species analyzed in the present study. Despite being very detailed, this author's osteological analysis was only descriptive, with no statistical evaluation of the variation among taxonomic units. In the present study, the geometric morphometric approach revealed significant variation in the shape of the skulls of the five *Leposternon* study species (*L. microcephalum*, *L. infraorbitale*, *L. polystegum*, *L. wuchereri*, and *L. scutigerum*), even after eliminating the effects of size. Significant differences in skull size were also found among species, in particular between *L. infraorbitale* and all the other study species. If supported by an adequate statistical approach, the skull may be a potential informative trait for phylogenetic studies which the objective is to differentiate some species within a morphotype.

The variation in skull shape represented by the wireframes derived from the Principal Components Analysis (Figure 6) is similar to that described by Barros-Filho (2000) in the same species. For example, the posterior contour of the occipital segment is semicircular, with a V-shaped insertion of the supraoccipital in *L. microcephalum*, whereas in *L. infraorbitale*, the contour is M-shaped, with the insertion of the supraoccipital in the central angle, with a marked obtuse angulation, and in *L. wuchereri*, the posterior contour of the occipital segment is shaped like a Greek letter ω. These patterns are consistent with the displacement of the landmarks on the posterior segment of the dorsal view of the skull in these species.

Leposternon infraorbitale was the species with the largest skull, which was associated with a marked constriction of the intermediate segment and a robust posterior portion. This indicates that this species seems to be able to exert a stronger bite and compression forces, but digs more slowly in comparison with the other *Leposternon* species, although biomechanical data will be needed to confirm this conclusion.

4.3 Biogeographical factors influencing shape

In the present study, the analysis of museum specimens limited the potential for the collection of more systematic data on the environment from which the specimens were collected, in particular the properties of the soil, such as humidity, porosity, and resistance to penetration. However, the geographic coordinates of each locality were used to determine 19 bioclimatic variables and the principal soil type (see Cáceres et al., 2013 and Bubadué et al., 2016). Based on data from the Food and Agriculture Organzation (FAO - http://www.fao.org/home/en/), gleysols are poorly drained, and are permanently or temporarily saturated with groundwater to a depth of 50 cm, while cambisols have good structural stability, water-holding capacity, internal drainage, and high porosity. Acrisols are equivalent to red-yellow podzolic soils, and have a poorly-developed structure that is porous under protective forest cover, but may form a hard surface crust after deforestation, with poor penetration of surface water. Ferralsols are equivalent to latosols, and have a very stable microstructure (pseudo-sand and pseudo-silt textures), good water-holding and transmission capacities, and low bulk density, although the surface is sealed and compacted.

This is consistent with the fact that the physical properties of the soil, in particular its resistance to penetration, will be related to its water content. A decrease in water content may result in an increase in compaction (Silveira et al., 2010), which in turn makes the soil more resistant to penetration (Pedroti et al., 2001; Almeida, 2008), specifically, by amphisbaenians. However, an excess of water in the soil may also be deleterious, as waterlogging will decrease oxygenation, which may force soil-dwelling organisms to the surface, especially during rainy periods during hot summers (Colli & Zamboni, 1999). The influence of precipitation and other bioclimatic variables on the skull shape of the *Leposternon* species may thus also support Corti et al. (1996), who concluded that soil type alone cannot account for all the change in shape, and that any cline will reflect a combination of ecogeographic variables.

The genus *Leposternon* is the only South American representative of the shovel-headed amphisbaenians, which are considered to be the most specialized for digging, allowing them to penetrate well-compacted soils (Gans, 1974; Kearney, 2003; Gans, 2005; Hohl et al., 2014). The results of the present study indicate that the shovel-headed forms are able to penetrate different soil types under a range of climatic conditions, especially considering the ample, but often sympatric distribution of the species studied here.

The occurrence of *L. microcephalum* in a wide range of soil types appears to be related to its ample distribution in South America. As *L. wuchereri*, *L. infraorbitale*, and *L. polystegum* also have relatively ample geographic ranges, it seems likely that they may also coincide with more soil types than indicated by the sample analyzed here. For example, Tavares (2015) recorded *L. polystegum* in Acrisols, Neosols, and Ferralsols (according to the WRB/FAO classification system). However, the only endemic species analyzed in the present study, *L. scutigerum*, occurred only in Acrisols, although it has also been observed in Podzolic soil by Gonçalves-Dias & Barros-Filho (1992).

Leposternon microcephalum thus appears to be more flexible in its ecological requirements than other *Leposternon* species. For instance, *L. polystegum* has a considerable ample distribution, but it is restricted to the soils of the Brazilian Northeast that may be similar to each other due the relatively arid climatic characteristics of the region. Overall, then, *L. microcephalum* not only occupies different soil types, but also different climatic conditions, which affect the structure of the soil. Due to its much more restricted distribution, *L. scutigerum* occupies a narrower range of soil types than of *L. microcpehalum*, even though some variation in soil types is found within its geographic range, in the state of Rio de Janeiro.

Leposternon polystegum was recorded at a locality where Ferralsols are associated with rainy winters. This soil type is one of the deepest found in the Brazilian semi-arid zone (Tavares, 2015) and the surface is sealed and heavily compacted. Productivity is also lower in regions with rainy winters, due to the decrease in sunlight, which results in a lower content of organic matter in the soil, reducing the retention of water in the superficial layers (Odum, 1988). As reduced water retention is known to increase the resistance of the soil to penetration (Almeida, 2008), the skull morphology of *L. polystegum* thus appears to be associated with harder, dry soils, especially in relation to the snout, which is large and rounded, and shovel-shaped in the dorsal view, while is the lateral view, it is projected in the anterior direction and elongated, with a discrete concave anterior border. The general appearance of the shape appears to be more “earth-dynamic”, indicating that it may facilitate, in particular, the initial penetration of the soil at the surface.

The results of the present study indicated that skull size was not related to any ecogeographical variable. The size of the shovel-headed skull is an important determinant of digging performance, given that the maximum width of the skull is closely related to excavation force (Navas et al., 2004). This indicates that excavation force alone does not determine where *Leposternon* can be found, but rather, the depth that the individual can penetrate into the soil, given the soil type or bioclimatic conditions found at a specific site. In *L. polystegum*, for example, Gomes et al. (2009) recorded males (head width (HW) = 8.2±1.4mm) deeper in the soil than females (HW = 6.5±1.3mm) or juveniles (HW = 5.2±0.2mm). In other words, individuals with different skull sizes can penetrate the soil to different depths.

ACKNOWLEDGMENTS

We thank all the museums and their respective curators for the loan of the specimens analyzed, and Stephen Ferrari for reviewing the English text. Financial support for this study was provided by the Prociência Fellowship Program/UERJ, FAPERJ (O. Rocha-Barbosa E-26/111.279/2014), CAPES/DS, and CNPq (O. Rocha-Barbosa: 302162/2012-7)

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APPENDIX I A full list of *Leposternon* specimens analyzed (*N* = 61). Legend: BA = Bahia; CE = Ceará; DC = Departamento Central; DCo = Departamento Cordillera; DF = Distrito Federal; DM = Departamento Misiones; GO = Goiás; PA = Pará; PR = Paraná; RJ = Rio de Janeiro; RO = Rondônia; SC = Santa Catarina; F = female; M = male; U = sex unknown; * = *Leposternon* sp according to Barros-Filho (2000).

Species	Collection Identity	Sex	Locality
L. infraorbitale	ZUFRJ 1313	F	Ilhéus, BA, Brazil

L. infraorbitale	ZUFRJ 1330	F	Ilhéus, BA, Brazil
L. infraorbitale	MNRJ 4034	F	Ilhéus, BA, Brazil
L. infraorbitale	MNRJ 4035	U	Itabuna, BA, Brazil
L. infraorbitale	MNRJ 4455	F	Ilhéus, BA, Brazil
L. infraorbitale	MNRJ 4456	F	Ilhéus, BA, Brazil
L. polystegum	MPEG 7145	M	Bragança, PA, Brazil
L. polystegum	MPEG 6559	M	Bragança, PA, Brazil
L. polystegum	MPEG 6649	M	Bragança, PA, Brazil
L. polystegum	MPEG 6650	U	Bragança, PA, Brazil
L. polystegum	MPEG 6671	F	Bragança, PA, Brazil
L. polystegum	MPEG 6624	F	Bragança, PA, Brazil
L. polystegum	MPEG 6604	M	Bragança, PA, Brazil
L. polystegum	MPEG 6634	M	Bragança, PA, Brazil
L. polystegum	MPEG 6673	F	Bragança, PA, Brazil
L. polystegum	MPEG 7223	F	Bragança, PA, Brazil
L. polystegum	MPEG 7600	M	Bragança, PA, Brazil
L. polystegum	MPEG 7602	F	Bragança, PA, Brazil
L. polystegum	UFC-L 1959	U	Maranguape, CE, Brazil
L. polystegum	UFC-L 2071	M	Ubajara, CE, Brazil
L. polystegum	ZUFRJ 527	M	Fortaleza, CE, Brazil
L. wuchereri	ZUFES 15/126	M	Mucuri, BA, Brazil
L. wuchereri	ZUFES 15/131	M	Mucuri, BA, Brazil
L. wuchereri	ZUFES 15/203	F	Mucuri, BA, Brazil
L. wuchereri	ZUFES 15/208	F	Mucuri, BA, Brazil
L. wuchereri	ZUFES 15/209	F	Mucuri, BA, Brazil
L. wuchereri	ZUFES 15/227	F	Mucuri, BA, Brazil
L. wuchereri	ZUFES 15/228	U	Mucuri, BA, Brazil
L. wuchereri	ZUFES 15/229	U	Mucuri, BA, Brazil
L. wuchereri	ZUFES 15/236	U	Mucuri, BA, Brazil
L. wuchereri	ZUFRJ 1555	U	Unknown
L. scutigerum	ZUFRJ 550	F	Itaboraí, RJ, Brazil
L. scutigerum	ZUFRJ 1399	M	Itaboraí, RJ, Brazil
L. scutigerum	ZUFRJ 1511	M	Saquarema, RJ, Brazil
L. scutigerum	UERJ 710	U	Rio de Janeiro, RJ, Brazil
L. scutigerum	MNRJ 4038	U	Itaboraí, RJ, Brazil
L. scutigerum	MNRJ 4490	M	Maricá, RJ, Brazil

L. microcephalum	MHNCI 3034	M	Adrianópolis, PR, Brazil
L. microcephalum	MHNCI 3538	M	Paranaguá, PR, Brazil
L. microcephalum	UnB 3663	F	Brasília (DF), GO, Brazil
L. microcephalum	UNNEC 511	M	Corrientes, Argentine
L. microcephalum	ZUFRJ 240	M	Bom Jesus do Itabapoana, RJ, Brazil
L. microcephalum	ZUFRJ 249	F	Rio de Janeiro, RJ, Brazil
L. microcephalum	ZUFRJ 462	M	Rio de Janeiro, RJ, Brazil
L. microcephalum	ZUFRJ 467	F	Rio de Janeiro, RJ, Brazil
L. microcephalum	ZUFRJ 468	M	Rio de Janeiro, RJ, Brazil
L. microcephalum	ZUFRJ 1001	U	Governador Celso Ramos, SC, Brazil
L. microcephalum	ZUFRJ 1317	M	Petrópolis, RJ, Brazil
L. microcephalum	ZUFRJ 1320	M	Petrópolis, RJ, Brazil
L. microcephalum	ZUFRJ 1321	M	Petrópolis, RJ, Brazil
L. microcephalum	ZUFRJ 1514	U	Unknown
L. microcephalum	ZUFRJ 1282	U	Unknown
L. microcephalum	ZUFRJ 975	U	Unknown
L. microcephalum	MHNCI 7347	F	Espigão d'Oeste, RO, Brazil
L. microcephalum	MNHNP 244	U	Departamento Caazapá, Paraguay
L. microcephalum	MNHNP 5114	M	Ayolas, DM, Paraguay
L. microcephalum	MNRJ 4789	F	Joinville, SC, Brazil
*L. microcephalum**	NMB 3818	M	Colonia Nueva Colombia, DCo, Paraguay
*L. microcephalum**	NMB 3819	M	Colonia Nueva Colombia, DCo, Paraguay
*L. microcephalum**	MNHNP 5112	M	Areguá, DC, Paraguay
*L. microcephalum**	NMB 3820	M	Colonia Nueva Colombia, DCo, Paraguay

Museums and Collections: **MHNCI:** Museu de História Natural do Capão da Imbuia; **MNHNP:** Museo Nacional de Historia Natural del Paraguay; **MNRJ:** Museu Nacional do Rio de Janeiro/UFRJ; **MPEG:** Museu Paraense Emílio Goeldi; **NMB:** Naturhistorisches Museum, Basel; **UERJ:** Coleção herpetologica da Universidade do Estado do Rio de Janeiro; **UFC:**Universidade Federal do Ceará; **UnB:** Universidade de Brasília - DF; **UNNEC:** Universidad Nacional del Nordeste – Corrientes (Argentine); **ZUFES:** Zoologia, Universidade Federal do Espírito Santo.; **ZUFRJ:** Zoologia, Universidade Federal do Rio de Janeiro.

APPENDIX II Description of the landmarks/semilandmarks used to describe the dorsal and lateral views of the *Leposternon* skulls.

Dorsal	1 – Tip of the snout (anteriormost point of the premaxilla); 2, 3, 15, and 16 – Distal contourn of the snout segment covering the maxilla and prefrontal; 4 and 14 – Distalmost point of the posterior lateral projection of the preforntals in the transversal crest; 5 and 13 – Anterior constriction of the intermediate segment in the parietals; 6 and 12 – Distal contourn of intermediate segment in the middle of the parietals; 7 and 11 – Distalmost point of the posterior segment in the projection of the paraoccipital processes; 8 and 10 – Extremity of the occipital crest; 9 – Posterior convavity of the supraoccipital.
Lateral	1 – Tip of the snout (anteriormost point of the premaxilla); 2, 3, and 4 – Anterior contourn of the snout segment; 5 – Apex of the transversal crest; 6 – Midpoint between landmarks 5 and 7; 7 – Dorsal curvature of the supraoccipital; 8 and 10 – Meeting between the posterior occiptal segment and the superior and inferior borders of the occipital condyle; 9 – Posteriormost point of the curvature of the occipital condyle; 11 – Antero-ventral limit of the X-element; 12 – Perpendicular point to the semilandmark 6 in the ventral contourn of the pterygoid; 13 – Ventral curvature between the anterior segment and palatal series, in the limits of maxilla and pterygoid; 14 – Extremity of the antero-ventral curvature of the maxilla.

APPENDIX III Raw data of the size information.

Species	Centroid Size	Log Centroid Size
Leposternon infraorbitale	4.967078639140912	1.6028318683213878
Leposternon infraorbitale	4.276061172261205	1.453032299014914
Leposternon infraorbitale	4.279700009277227	1.4538829158468287
Leposternon infraorbitale	3.7481820222234403	1.3212709283579298
Leposternon infraorbitale	3.4678291627074134	1.243528796663239
Leposternon polystegum	2.262553118861342	0.8164938742661059
Leposternon polystegum	1.400304850353464	0.33668996316954203
Leposternon polystegum	1.8679120227143997	0.6248212416495842
Leposternon polystegum	1.4416479836486393	0.36578689231289735
Leposternon polystegum	2.030339688140971	0.7082031131145055
Leposternon polystegum	1.4520736166861081	0.37299261531443695
Leposternon polystegum	1.5584747055435926	0.44370759009264377
Leposternon polystegum	2.2948362106972633	0.8306614730777969
Leposternon polystegum	1.6610431912975276	0.5074458334824412
Leposternon polystegum	1.9531621444792255	0.6694496717351534
Leposternon polystegum	2.0520039696548773	0.7188168618363192
Leposternon polystegum	1.9209466275674802	0.652818099729171
Leposternon polystegum	2.37542018596579	0.8651743422446835
Leposternon polystegum	1.8865025569985152	0.6347246158406126
Leposternon polystegum	1.9999508380162594	0.6931225992659574
Leposternon wuchereri	1.9745978467672856	0.6803647557293515
Leposternon wuchereri	1.7837086059593295	0.5786946833500572
Leposternon wuchereri	1.7092410701066092	0.5360494533468151
Leposternon wuchereri	1.5576605770513474	0.4431850655955104
Leposternon wuchereri	1.865713784025148	0.623643705893704
Leposternon wuchereri	1.5836098160425873	0.4597069347925038
Leposternon wuchereri	1.5235329901723496	0.42103197340829146
Leposternon wuchereri	1.5305778300389936	0.4256453307983823
Leposternon wuchereri	1.5884841463571486	0.46278019441773055
Leposternon wuchereri	1.347753293836334	0.29843897929867386
Leposternon scutigerum	2.0173115339459535	0.7017657011582824
Leposternon scutigerum	2.096227340010665	0.7401392243225752
Leposternon scutigerum	2.3543717579000636	0.8562739224098892
Leposternon scutigerum	2.26785928713378	0.8188363412308035

Leposternon scutigerum	1.7813545845340393	0.5773740774355898
Leposternon scutigerum	1.698182373534421	0.5295584870300365
Leposternon microcephalum	2.586601109449655	0.9503447009209187
Leposternon microcephalum	2.311786173998839	0.8380204612384826
Leposternon microcephalum	3.089494063154141	1.1280073438967697
Leposternon microcephalum	1.796125727082861	0.5856319713735326
Leposternon microcephalum	2.0269698803073024	0.7065420081129425
Leposternon microcephalum	2.435909749573427	0.890320300546156
Leposternon microcephalum	1.6475857035519998	0.499311006406915
Leposternon microcephalum	2.784807420215703	1.024178722053234
Leposternon microcephalum	1.9818843138710034	0.6840480658243785
Leposternon microcephalum	1.6989418992360181	0.5300056450691703
Leposternon microcephalum	2.752905281179855	1.0126568198947186
Leposternon microcephalum	2.9256488929177635	1.0735162998127064
Leposternon microcephalum	2.666876675423975	0.9809080031948398
Leposternon microcephalum	2.4622043813212375	0.9010570386633243
Leposternon microcephalum	1.5900004384879702	0.46373429201069977
Leposternon microcephalum	1.4501466373948186	0.37166468055707236
Leposternon microcephalum	3.0558132783868683	1.1170457694332958
Leposternon microcephalum	1.974181154802036	0.6801537072170745
Leposternon microcephalum	2.312531482863693	0.8383428045230974
Leposternon microcephalum	1.9353682764174367	0.6602976321024232
Leposternon microcephalum	1.4904067903273925	0.39904909633847035
Leposternon microcephalum	1.7872252497936572	0.5806642773628421
Leposternon microcephalum	1.8702916938777225	0.626094404732807

APÊNDICE C – Body and skull morphometric variations between two shovel-headed species of Amphisbaenia (Reptilia: Squamata) with morphofunctional inferences on burrowing.

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ABSTRACT

Background. Morphological descriptions comparing *Leposternon microcephalum* and *L. scutigerum* have been made previously. However, these taxa lack of a formal quantitative morphological characterization and comparative studies suggest that morphology and burrowing performance are related. The excavatory movements of *L. microcephalum* have been described in detail. However, there is a lack of studies comparing locomotor patterns and/or performance among different amphisbaenids sharing the same skull shape. This paper presents the first study of comparative morphometric variations between two closely related amphisbaenid species, *L. microcephalum* and *L. scutigerum,* with functional inferences on fossorial locomotion efficiency.

Methods. Inter-specific morphometric variations were verified through statistical analyses of body and cranial measures of *L. microcephalum* and *L. scutigerum* specimens. Their burrowing activity was assessed through X-ray videofluoroscopy and then compared. The influence of morphological variation on the speed of digging was tested among *Leposternon* individuals.

Results. *Leposternon microcephalum* and *L. scutigerum* are morphometrically distinct species. The first is shorter and robust with a wider head while the other is more elongated and slim with a narrower head. They share the same excavatory movements. The animals analyzed reached relatively high speeds, but individuals with narrower skulls dug faster. A negative correlation between the speed and the width of skull was determined, but not with total length or diameter of the body.

Discussion. The morphometric differences between *L. microcephalum* and *L. scutigerum* are in accord with morphological variations previously described. Since these species performed the same excavation pattern, we may infer that closely related amphisbaenids with the same skull type would exhibit the same excavatory pattern. The negative correlation between head width and excavation speed is also observed in others fossorial squamates. The robustness of the skull is also related to compression force in *L. microcephalum*. Individuals with wider heads are stronger. Thus, we suggest trade-offs between excavation speed and compression force during burrowing in this species.

INTRODUCTION

Amphisbaenians are fossorial reptiles with an elongated and cylindrical body shape (Gans, 1969) and, except the three *Bipes* species, all (nearly 200 species, Uetz et al., 2016) are limbless. They are a monophyletic group within Squamata (Gans, 1969; Kearney, 2003; Kearney & Stuart, 2004).

A strongly ossified and compact skull is common to all amphisbaenids. However, their heads may show four different morphological patterns: spade-head, keel-head, round-head, and shovel-head (Kearney, 2003). The “shovel” type, shared by the genera *Rhineura* (Rhineuridae, North America), *Dalophia* and *Monopeltis* (Amphisbaenidae, Africa), and *Leposternon* (Amphisbaenidae, South America), is considered the most specialized for digging (Gans, 1974, 2005; Kearney, 2003; Hohl et al, 2014). This means that these animals are able to penetrate more easily into highly compacted soils reaching greater depths.

Currently, there are ten recognized *Leposternon* species (Ribeiro et al., 2011; Ribeiro et al., 2015). The species *Leposternon microcephalum* has a widespread distribution, occurring in different regions of Brazil, as well as in Bolivia, Paraguay, Argentina, and Uruguay (Perez & Ribeiro, 2008; Ribeiro et al., 2011). The widespread distribution of *L. microcephalum* throughout South America is associated with recognized geographic morphological variation (Barros-Filho, 2000; Gans & Montero, 2009), including Paraguayan individuals that were suggested as being a different species by Barros-Filho (2000). In the State of Rio de Janeiro, Brazil, *L. microcephalum* may occupy different soil types with different degrees of compaction and depth (Gonçalves-Dias & Barros-Filho, 1992). On the other hand, *L. scutigerum* has a more restricted distribution, endemic to the State of Rio de Janeiro, Brazil (Barros-Filho, 1994; Rocha et al., 2009), and occupies a specific soil type, less compacted and shallower than those occupied by *L. microcephalum* (Gonçalves-Dias & Barros-Filho, 1992). Also, it is included as Endangered (status EN) on the IUCN Red List of Threatened Species (Colli et al., 2016), and on Brazil’s National Red List (see Portaria MMA nº 444, 17 December, 2014).

Morphological differences between individuals of *L. microcephalum* and *L. scutigerum* from the State of Rio de Janeiro include the number of annuli and vertebrae, snout-vent length, cephalic and pectoral shields configuration (Gans, 1971), and skull descriptive morphology (Barros-Filho, 2000; Montero & Gans, 2009). Among the osteological differences, the vertebrae numbers are 93-103 for *L. microcephalum* and 119-123 for *L. scutigerum* (Gans, 1971). Barros-Filho (2000) described the general anatomy of the skull of these species including features that we consider related to the excavation process. The skull of *L. microcephalum* can be distinguished from *L. scutigerum* by the diamond-shaped outline of the anterior view of the facial region; angulations between facial and medial skull regions tending to less than 120^o (mean value = 118.8^o); the largest width of the facial region being conspicuously shorter than the width of the occipital region; the prefrontal bones not having any conspicuous lateral expansions; and the facial region not being especially expanded laterally at the transverse crest line (between facial and medial regions) (Barros-Filho, 2000). On the other hand, in *L. scutigerum*, the anterior view of the facial region has a triangular-shaped outline; the average angulation between facial and medial skull regions is 121.6^o; the largest width of the facial region is very close to the width of the occipital region; the prefrontal bones present characteristic lateral expansions; and there is a characteristic lateral expansion at the transverse crest line (Barros-Filho, 2000).

Despite the good qualitative description of morphological variations present in the literature, there are no body and/or skull morphometric differences supported by statistical approaches for these species. Furthermore, the morphofunctional consequences of the morphological variations in *L. microcephalum* and *L. scutigerum* remain unknown.

Fossorial animals have to be able to travel along an existing tunnel, burrow or extend existing tunnel systems, and leave the surface by penetrating the soil (Gans, 1978). The high degree of specialization of the amphisbaenians is well expressed in the speed and effectiveness with which they burrow tunnel systems and penetrate the substrate, even in fairly hard soils (Gans, 1978).

The excavation patterns of the Amphisbaenia range from random movements of the head and lateral movements to “shovel” and “screw” movements, which vary according to the four head types (Gans, 1974). Shovel-headed amphisbaenians excavate through “shovel” movements of the head, which is considered the most specialized digging pattern (Gans, 1974). In fact, morphological features and underground locomotor performance seem to be related. For instance, body and head size and strength directly affect the ability of fossorial squamates to penetrate the substrate and move inside their galleries (Gans, 1974; Navas et al., 2004). Navas et al. (2004) postulated that *L. microcephalum* individuals with narrower heads dig faster than those with a wider head or more robust body. Gans (1978) suggested that the osteological reinforcement of the transverse crest, together with the discrete lateral projection of the otic capsules may be important for improving excavation efficiency. Furthermore, Vanzolini (1951) and Barros-Filho (1994) associated the fusion of cephalic and pectoral shields with the reduction of friction with the substrate, facilitating the underground displacement of the animal.

In the last 60 years, the locomotor behavior of *L. microcephalum* has been incidentally studied through visual observations (Kaiser, 1955), motion pictures of the external body (Navas et al., 2004), and the videofluoroscopy technique (Barros-Filho et al., 2008; Hohl et al., 2014). The most recent description of the excavatory cycle of *L. microcephalum* is presented as: (1) initial static position with the gular and anterior body regions lying over the tunnel floor; (2) retreating and downward bending of the head, with tip of snout touching the floor substrate; (3) a continuous upward and forward head movement, which compacts the substrate granules against the tunnel roof, while the pectoral region compresses the tunnel floor. This is followed by the dropping of the head, returning to the initial static position (Barros-Filho et al., 2008; Hohl et al., 2014).

Not only there are few studies of the underground locomotion of *L. microcephalum,* but all of them faced the problem of limited samples (Kaiser, 1955; Navas et al., 2004; Barros-Filho et al., 2008; Hohl et al., 2014). In fact, there are no studies of other species of *Leposternon*. Furthermore, there is a lack of studies comparing locomotor patterns and/or performance among different amphisbaenid species. Similarly, the review of literature shows that quantitative studies inferring the influence of morphological variation on the fossorial locomotor performance of amphisbaenids are scarce (e. g. López et al., 1997; Navas et al., 2004).

Given that there are notable morphological variations between *L. microcephalum* and *L. scutigerum*, we hypothesized that body and skull morphometric variation is also evident. Therefore, such variation could impact locomotor performance. Considering that *L. microcephalum* and *L. scutigerum* share the same general shovel-headed pattern, they should have similar excavatory movements. Furthermore, based on the observations of Navas et al. (2004) that individuals of *L. microcephalum* with narrower heads exert less force but dig faster than those with a wider head or more robust body, we believe that *Leposternon* individuals with narrower skulls and bodies may dig faster.

The main objective of the present study is to characterize the morphometric variations in body and skull between *L. microcephalum* and *L. scutigerum*, providing morphofunctional inferences on burrowing. We also describe the excavatory pattern and performance of *L. scutigerum*, comparing its locomotor traits with *L. microcephalum*; and we verify the relation between the length and robustness of the body and skull with the burrowing speed among *Leposternon* individuals.

MATERIAL AND METHODS

Morphometric analysis

A total of 26 adult *Leposternon* specimens were measured (*L. microcephalum* Wagler, 1824 *n* = 14; *L. scutigerum* (Hemprich, 1820) *n* = 12). The sample included eight live individuals used in the locomotion analyses and 18 specimens from scientific collections deposited at Museu Nacional and Departamento de Zoologia of the Universidade Federal do Rio de Janeiro (UFRJ). The specimens from scientific collections were prepared, after we took the body measures, through exposure to *Dermestes* sp (Coleoptera, Insecta) larvae or maceration in hydrogen peroxide (H_2O_2) (see Barros-Filho, 2000). Individuals with a length of more than 150 mm were considered adults (Gans, 1971), and no sexual dimorphism was found for the variables analyzed. *Leposternon microcephalum* has some morphological variation along its geographic distribution in South America. Our analysis included only specimens from Rio de Janeiro, Brazil, therefore, we expected minor differences could exist considering particular ecotypes of this species. Identification of all specimens can be found in Table 1.

Table 1: Morphometric data of *L. microcephalum* (*n* = 14) and *L. scutigerum* (*n* = 12) specimens. All measures are in millimeters.

Specimens	Species	TL	D	CBL	MW	TCW	MWR
1/2012	*L. microcephalum*	360	13.7	20.8	12.5		
3/2012	*L. microcephalum*	333	15	22.3	9		
4/2012	*L. microcephalum*	294	16.2	16.2	12.5		
5/2012	*L. microcephalum*	354	16	18.7	9		
8/2012	*L. microcephalum*	370	17.5	22.3	12.3		
9/2012	*L. microcephalum*	364	20	20.5	13.7		
10/2012	*L. microcephalum*	298	16.2	18.5	10		
1/2017	*L. scutigerum*	428	11.7	14.8	6.5		
ZUFRJ240	*L. microcephalum*	260	13.1	14.3	8.2	5.1	5.5
ZUFRJ249	*L. microcephalum*	430	18.4	16.8	9.6	7	7.3
ZUFRJ285	*L. microcephalum*	463	20.6	18.9	11.1	7.3	8
ZUFRJ467	*L. microcephalum*	466	19.1	19.1	10.7	7.3	8.1
ZUFRJ468	*L. microcephalum*	326	10.5	14.2	7.9	4.9	5.2
ZUFRJ1320	*L. microcephalum*	490	16.4	20	12.7	7.5	8
ZUFRJ1321	*L. microcephalum*	425	17.6	18.8	10.2	7.2	7.7
MNRJ4036	*L. scutigerum*	481	12.4	17.5	9.8	8	8.5
MNRJ4037	*L. scutigerum*	489	15.3	17.9	9.4	7.8	8.1
MNRJ4038	*L. scutigerum*	408	10.4	14.7	7.4	5.9	5.9
MNRJ4458	*L. scutigerum*	490	12.5	17.8	9.7	8.2	8.3
MNRJ4490	*L. scutigerum*	390	10.7	14.1	7.4	5.6	5.6
MNRJ4791	*L. scutigerum*	443	10.7	16.8	8.8	7.3	7.3
ZUFRJ550	*L. scutigerum*	375	10.7	14.5	7.3	5.8	6.3
ZUFRJ1399	*L. scutigerum*	413	11.8	14.8	7.8	6	6.5
ZUFRJ1401	*L. scutigerum*	467	13.7	18	9.4	8.3	8.4
ZUFRJ1417	*L. scutigerum*	462	14.9	17.6	9.7	7.6	8
ZUFRJ1511	*L. scutigerum*	514	12.8	16.7	8.8	7.2	7.8

Notes: Body measures: TL = total length; D = diameter; Skull measures: CBL = condylo-basal length; MW = maximum width; TCW = tranversal crest width; MWR = maximum width of the rostral region; Scientific Collections: MNRJ = Museu Nacional da Universidade Federal do Rio de Janeiro; ZUFRJ = Zoologia, Universidade Federal do Rio de Janeiro. The first nine animals were recorded and released back into the wild and are identified by the number of specimen/year of recording.

Body measurements were taken using a measuring tape (in mm scale) whereas skull measurements were recorded with a digital caliper (model Pro-Max, Fowler-NSK scale 0.01mm). The skull measurements were: condylo-basal length (CBL); maximum width (MW), situated at the occipital region covering the optical capsules; the transverse crest width (TCW); and maximum width of the rostral region (MWR) (Fig. 1). The body measurements included: the total body length (TL) and diameter (D) in the middle of the body. The terminology of cranial structures follows Gans & Montero (2009). The skull measurements of the live individuals were taken using X-ray records through the software Tracker v. 4.96. The distance between lead pieces in the wall of the terrarium was used as scale (5 cm). For this sample, it was not possible to measure the TCW and MWR.

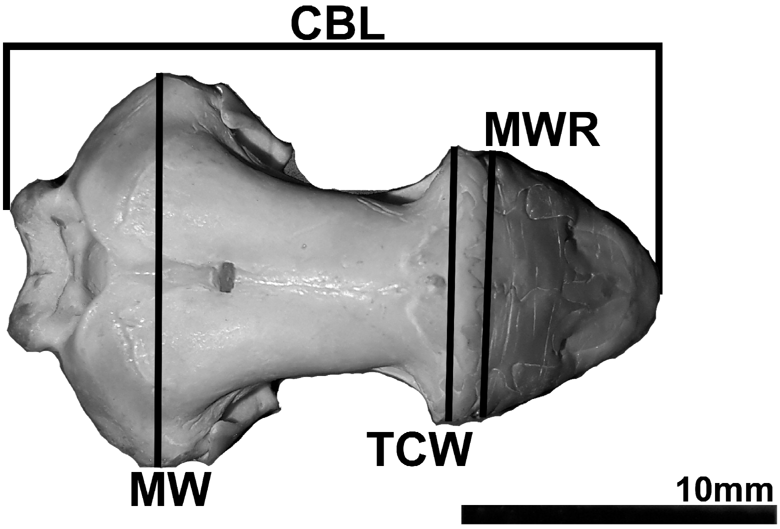

Figure 1: Photography of the dorsal view of a *Leposternon microcephalum* skull showing the measures taken. CBL = condylo-basal length; MW = maximum width; TCW = transverse crest width; MWR = maximum width of the rostral region.

The frequency distributions of the morphometric variables were tested for normality (Shapiro-Wilk’s T) and homoscedasticity (Levene), as well as, skewness and kurtosis. The Wilcoxon-Mann-Whitney *U*-Tests were used to test intra- and inter-specific morphometric variations among *L. scutigerum* and *L. microcephalum* specimens by comparing body and skull measurements. Comparisons between groups were performed through Principal Component Analysis (PCA) as a data exploratory method, over the standardized values of the original variables considered (TL, D, CBL and MW), to verify their trends of variation and the group distribution in their multivariate morphological space (Zarr, 1999). Statistical analyses were performed with Statistica v.8.0 (Statsoft, 2008).

Locomotion analysis

Eight adult individuals of the genus *Leposternon* from Rio de Janeiro City were analyzed (*L. microcephalum n* = 7; *L. scutigerum n* = 1). *Leposternon scutigerum* is an elusive species. The individuals of *L. microcephalum* were collected, recorded and analyzed by Hohl et al. (2014). Field collections were approved by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), environmental agency of the Brazilian government, with a permanent license number for collecting zoological material (15337), since May 28th, 2008. This locomotor behavior study was approved by the Ethics Committee for the Care and Use of Experimental Animals of the Instituto de Biologia Roberto Alcantara Gomes (CEUA/IBRAG/015/2017). The individuals are identified by number of specimen/year of recording in Table 1.

Locomotion was recorded using videofluoroscopy. This technique, based on X-ray recording, has been used in behavioral studies of fossorial species of amphisbaenians (Barros-Filho et al., 2008; Hohl et al., 2014) and caecilians (Summers and O'Reilly, 1997; Measey and Herrel, 2006, Herrel and Measey 2010, 2012).

Before recording, *L. scutigerum* was maintained in the laboratory at the Laboratório de Zoologia de Vertebrados -Tetrapoda (LAZOVERTE), Universidade do Estado do Rio de Janeiro (UERJ) under the same conditions presented by Hohl et al. (2014), i. e., kept in a plastic box containing humid humus-rich soil, and feed with earthworms once a week. The X-rays were performed at the Hospital Universitário Pedro Ernesto (HUPE/UERJ), Rio de Janeiro, with a Toshiba videofluoroscopy machine (Toshiba Ultimax from Toshiba Medical Systems) that films at 30 frames per second with calibration of 200 mA and 40 kV. In addition, following the same procedures used by Hohl et al. (2014) to film individuals of *L. microcephalum*, *L. scutigerum* was kept in a glass terrarium filled with dry/loose semolina, and with lead markers placed on the outer face of the wall terrarium. More details about the recording procedure can be found in Hohl et al. (2014). After filming, the animal was released back into the wild.

A total recording time of 7 min and 40 s containing 32 motion sequences of *L. scutigerum* was analyzed frame by frame using the Toshiba Daicom Viewer software. The excavatory pattern was analyzed taking into account the details of the body postures and movements performed. The locomotor performance was evaluated through the distance covered, duration of the movement, speed and frequency of cycles performed, according to Hohl et al. (2014).

Despite all *Leposternon* individuals sharing the same skull type, “shovel”, the sample contained two species. Thus, the excavatory pattern of *L. scutigerum* (*n* = 37 cycles) was determined and compared inter-specifically with the three-step gait pattern exhibited by *L. microcephalum* (*n* = 132 cycles), according to Barros-Filho et al. (2008) and Hohl et al. (2014).

The small sample of individuals hindered more comprehensive inter-specific statistical comparisons. Therefore, we evaluated the degree of variation observed in the video footage among the individuals recorded as a starting point to estimate possible differences between locomotor performance in *L. scutigerum* and *L. microcephalum*. The three fastest excavatory cycles (i.e. in which the animals reached highest speeds) performed by each individual (*n* = 7 *L. microcephalum* and *n* = 21 excavatory cycles; *n* = 1 *L. scutigerum* and *n* = 3 excavatory cycles) were quantified according to the locomotory parameters proposed.

Test of the relation between morphometric variables and burrowing speed

The influence of morphological variations on locomotor performance was assessed through Multiple Linear Regressions. The speed, expressing the degree of tunneling specialization (Gans, 1978), was used as the dependent variable to be regressed against body and skull measurements considered important for excavation ability according to Gans (1974) and Navas et al. (2004): total body length and diameter, and the maximum width of skull (independent variables). The analyses were performed with Statistica v.8.0 (Statsoft, 2008) first adding only *L. microcephalum* individuals, and then inserting the one individual of *L. scutigerum*.

RESULTS

Morphological variation

Inter-specific differences between *L. microcephalum* and *L. scutigerum* were observed in total length (TL) (U = 33.5, $p < 0.01$), diameter of the body (D) (U = 17.5, $p < 0.001$), condylo-basal length (CBL) (U = 34.5, $p < 0.01$), and maximum width of the skull (MW) (U = 27.0, $p < 0.01$). However, there were no statistical differences in the transverse crest width (TCW) or maximum width of the rostral region of the skull (MWR). Statistics are given in Table 2.

Table 2: Comparative analyses of body and skull morphometric data between *L. scutigerum* and *L. microcephalum*.

	L. microcephalum	*L. scutigerum*	C. V. (%) Lm/Ls	*U*-Test *P* value
TL	371.1 ± 69.1 (*N* = 15)	446.6 ± 44.1 (*N* = 12)	18.6 / 9.9	<0.01 **
D	16.45 ± 2.77 (*N* = 14)	12.31 ± 1.65 (*N* = 12)	16.8 / 13.4	<0.001 **
CBL	18.67 ± 2.56 (*N* = 14)	16.27 ± 1.55 (*N* = 12)	13.7 / 9.5	<0.01 **
MW	10.67 ± 1.84 (*N* = 14)	8.5 ± 1.16 (*N* = 12)	17.2 / 13.6	<0.01 **
TCW	6.61 ± 1.11 (*N* = 7)	7.06 ± 1.04 (*N* = 11)	16.8 / 14.7	>0.05 ns
MWR	7.11 ± 1.24 (*N* = 7)	7.34 ± 1.07 (*N* = 11)	17.4 / 14.6	>0.05 ns

Notes: C. V. = Coefficient of Variation; Lm = *Leposternon microcephalum*; Ls = *Leposternon scutigerum*. Values are presented by means and standard deviation, in millimeters.

The PCA analysis indicated two main axes of group differentiation: PC1 and PC2 explained, respectively, 60.63% and 25.03% of the variation. On PC1, the variables D, CBL, and MW loaded strongly and negatively. On PC2, the total length of the body loaded negatively and strongly (Table 3).

Table 3: The PCA loadings of the variables for the two main axis of variation (PC1 and PC2). Larger absolute values indicate more influential traits on the multivariate space. The higher loadings in each axis were highlighted in bold. Eigenvalues and cumulative percentage of variation explained are also indicated.

	PC 1	PC 2
TL	-0.06223	**-0.99702**
D	**-0.88608**	0.03308
CBL	**-0.89178**	-0.04454
MW	**-0.91613**	0.07889
Eigenvalue	2.423571	1.000637
Cumulative %	60.6308	85.6638

According to the PCA scatterplot (Fig. 2), *L. scutigerum* specimens occupied higher positive scores on PC1 and negative scores on PC2, tending to exhibit a greater elongated and slim body shape with a narrower head. On the other hand, *L. microcephalum* specimens occupied greater negative scores on PC1 and positive scores on PC2, featuring a shorter and robust body shape with a wider head.

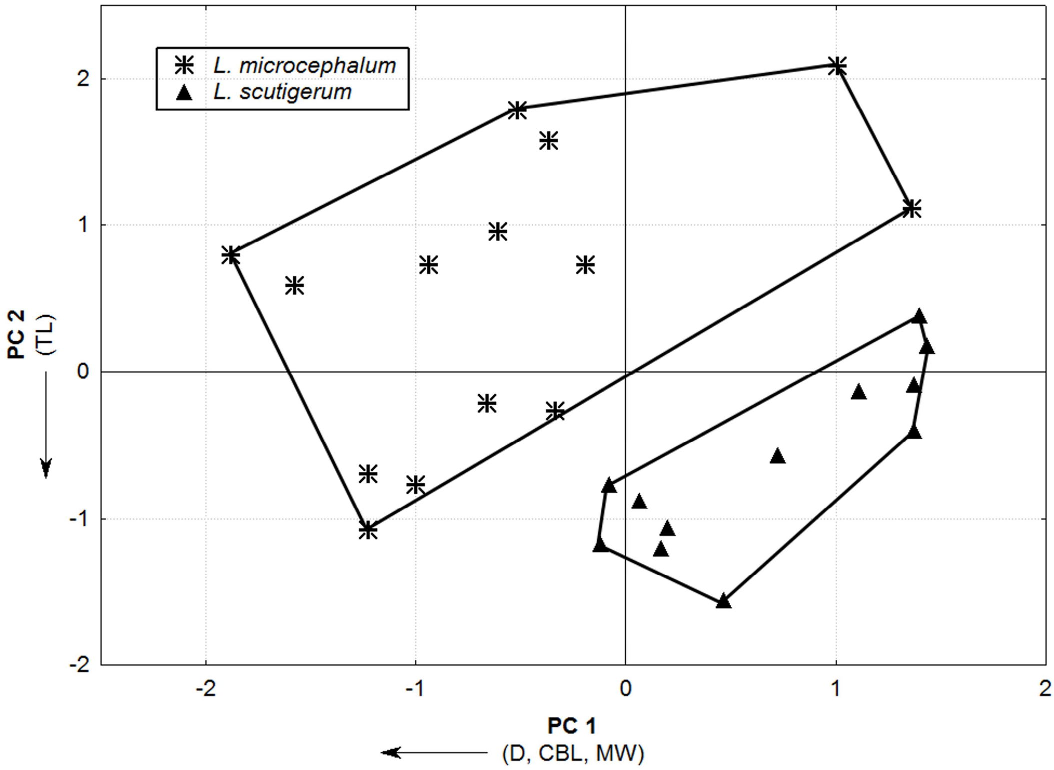

Figure 2: Principal Component Analysis scatterplot showing the distribution of *L. microcephalum* and *L. scutigerum* specimens on the multivariate space through the two main axis of variation (PC1 and PC2). Arrows indicate the direction of the correlation of each variable with the different PCs.

Locomotor variation

A detailed analysis of the movement, through visual observation of X-ray images, revealed that the three-step excavatory cycle of *L. scutigerum* seemed to be the same as *L. microcephalum*. Barros-Filho (2008) and Hohl et al. (2014) described this movement as: (1) initial static position with the gular and anterior body regions lying over the tunnel floor; (2) retreating and downward bending of the head, with tip of snout touching the floor substrate; (3) a continuous upward and forward head movement, which compacts the substrate granules against the tunnel roof, while the pectoral region compresses the tunnel floor. This is followed by the dropping of the head, returning to the initial static position.

According to the locomotor performance data, *L. scutigerum* constructed galleries with an average speed of 0.465 cm s^{-1} (± 0.2), travelling 0.58 cm (± 0.15) in

1.26 s (± 0.43) seconds, and performed almost a complete excavatory cycle each second (0.8 ± 0.3 Hz). The three fastest excavatory cycles of *L. scutigerum* showed that it constructed galleries with an average speed of 0.757 cm s^{-1} (± 0.06), travelling 0.73 cm (± 0.11) in 0.83 s (± 0.23) seconds, and performed a complete excavatory cycle each second (1.06 ± 0.17 Hz). During the three fastest excavatory cycles, *L. microcephalum* was able to reach an average speed of 0.350 cm s^{-1} (± 0.09), travelling 0.59 cm (± 0.18) in 1.77 s (± 0.59), and performed about a half complete excavatory cycle each second (0.61 ± 0.15 Hz).

The mean values and SDs of the locomotor performance variables suggest that some inter-specific differences may exist between the species. *Leposternon scutigerum* presented higher averages for speed, travel distance and frequency of excavatory cycles, and lower values for cycle duration in relation to those found for *L. microcephalum*. On the other hand, the values of the coefficients of variation of the locomotor variables indicated some constancy in the species' performances along the intra-specific footages. Therefore, despite the sample size limitation, based on this functional approach to the excavatory performance of the species, we hypothesized that *L. scutigerum* could be a better excavator than *L. microcephalum,* in the sense of speed of soil penetration and gallery construction. Descriptive statistics values are presented in Table 4.

Table 4: Descriptive statistical values of the fastest excavatory cycles performed by *L. scutigerum* (three cycles of one individual) and *L. microcephalum* (21 cycles of seven individuals).

	L. microcephalum	*L. scutigerum*	C. V. (%) Lm/Ls
Speed (cm s^{-1})	0.350 ± 0.09	0.757 ± 0.06	25.8 / 8
Distance (cm)	0.59 ± 0.18	0.73 ± 0.11	30.6 / 15.4
Time (s)	1.77 ± 0.59	0.83 ± 0.23	33.3 / 28
Frequency (Hz)	0.61 ± 0.15	1.06 ± 0.17	24.2 / 16.6

Notes: C. V. = Coefficient of Variation; Lm = *Leposternon microcephalum*; Ls = *Leposternon scutigerum*. Values are presented by means and standard deviation.

Test of the relation between morphometric variables and burrowing speed

When the analysis was restricted to *L. microcephalum* individuals, linear regression demonstrated a negative correlation between the speed (dependent variable) and the width of skull (independent variable) (r^2 = 0.613; p = 0.037) (Fig.3A), but not with total length (r^2 = 0.001; p = 0.944) or diameter of the body (r^2 = 0.08; p = 0.54). When inserting the unique specimen of *L. scutigerum,* the analysis revealed a stronger and significant negative correlation between speed and width of skull (r^2 = 0.798; p = 0.003) (Fig.3B). Relations with total length (r^2 = 0.30; p = 0.153) or diameter of the body (r^2 = 0.42; p = 0.079) remained non-significant.

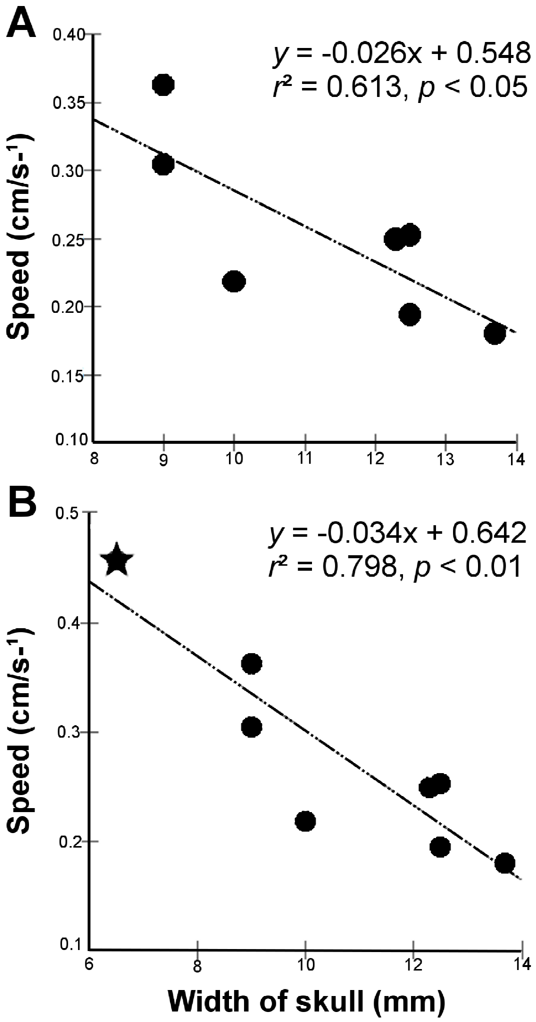

Figure 3: Excavatory cycle speed as a function of width of skull in *Leposternon* individuals. (A) Analysis restricted to *L. microcephalum*. (B) Analysis after insertion of *L. scutigerum*, represented by the star.

DISCUSSION

Inter-specific morphometric variation

Anatomical differences between *L. microcephalum* and *L. scutigerum* from Rio de Janeiro State are well known (Gans, 1971; Barros-Filho, 2000; Gans & Montero, 2009). The initial hypothesis that body and skull morphometric differences exist was corroborated. Significant differences between these two species in total length and diameter of the body, and maximum width of skull exist.

As suggested by the great number of vertebrae, *L. microcephalum* with 93-103 vertebrae and *L. scutigerum* with 119-123 (Gans, 1971), *L. scutigerum* presented a more elongated body. According to Barros-Filho (2000), the largest width of the facial region also covering the transverse crest of *L. scutigerum* is very close to the maximum width of the skull at the occipital region. This configuration is different for *L. microcephalum*, in which the largest width of the facial region is shorter than the width of the occipital region. Based on this difference, that author concluded that *L. scutigerum* "has a very characteristic skull, specially the lateral expansion of the facial region, at the transverse crest line". However, our results indicated statistical differences between these species for maximum width of skull (occipital region), but not for transverse crest width or maximum width of the rostral region (facial region). Thus, the allometric relation between facial and occipital regions of these species proposed by Barros-Filho (2000) is more related to occipital differences than facial ones.

Inter-specific comparisons of excavatory behavior

Barros-Filho et al. (2008) discussed the efficiency of videofluoroscopy as a methodology for analyzing locomotor behavior in fossorial squamates. They concluded that among the methodologies previously used (e. g. visual observations and motion pictures of the external body), videofluoroscopy was the most efficient in analyzing excavatory cycles. Considering that the present study also used videofluoroscopy as the methodology to access the fossorial locomotion of *L. scutigerum*, and replicated the movie set used by Barros-Filho et al. (2008) and Hohl et al. (2014) (i. e. the same glass terrarium marked with lead pieces and filled with dry/loose semolina), we conclude that the comparison results of locomotor pattern and performance between *L. scutigerum* and *L. microcephalum* were not influenced by methodological factors.

Leposternon scutigerum performed, in horizontal and vertical directions, the same three-step excavatory cycle first described by Barros-Filho et al. (2008) for *L. microcephalum*, reinforced by Hohl et al. (2014), as a retreating and downward bending of the head from an initial static position followed by an upward and forward head movement. This result suggests some partial support for the initial hypothesis that morphologically similar amphisbaenids would exhibit the same excavatory pattern. The results also suggests some support for the assumption of Barros-Filho et al. (2008) that, in spite of specific differences among the shovel-headed amphisbaenid species, the excavatory pattern was the same for all of those species, based on the fact that the shovel-head is, apparently, a convergence among the group (cf. Gans, 1974).

However, despite sharing the same skull type and performing three-step excavatory pattern, our quantitative approach of locomotor performance of *L. scutigerum* and *L. microcephalum* suggested differences in all parameters analyzed (travel distance, excavatory cycle duration, speed, and frequency). According to Hohl et al. (2014), *L. microcephalum* was able to build a gallery of 12 cm in length in 1 min. Our results showed that this species was able to build a gallery of 21.6 cm in length in 1 min (mean values based on the three fastest cycles), whereas the single individual of *L. scutigerum* studied was able to build a gallery of 46.4 cm in length in 1 min, i.e. more than twice, on average. Despite the lack of a formal statistical significance test, these values suggest that *L. scutigerum* may be a faster digger than *L. microcephalum*.

Morphological features and locomotor performance

Gans (1974) and Navas et al. (2004) postulated that body, head size and strength directly affect the speed of fossorial squamates to penetrate the substrate and move inside their galleries. However, they only reported it qualitatively. Navas et al. (2004), although relating the robustness of the head with the compression force, did not obtain the speed with which the animals excavated to associate with head width. According to our quantitative approach these previous statements seem partially true. Body dimensions (total length or diameter) seem to be not related to the increased speed of excavation. Despite the p-value above the alpha = 0.05, it seems to be a trend of correlation between body diameter and speed. We are positive that a larger sample could bring more assurance to this hypothesis. However, regression analysis showed that the width of skull is related to the speed of excavation (individuals with slender skulls seem to be faster). The finding, that head width is negatively correlated with excavation speed, is in agreement with previous studies. López et al. (1997) showed that individuals of *Blanus cinereus* with narrower, longer heads burrow faster. Vanhooydonck et al. (2011) showed that in burrowing skinks *Acontias percivali*, individuals with narrow heads were able to dig faster than broader-headed ones. Similar to our results, Vanhooydonck et al. (2011) also concluded that the speed in soil penetration is only predicted by the head width, curiouslly getting similar statistical values (*L. microcephalum* - $r^2 = 0.613$, $p = 0.037$; *Acontias percivali* - $r^2 = 0.64$, $p = 0.03$).

The robustness of the skull has been demonstrated to be an important characteristic to improve soil penetration. Beyond being associated with excavation speed, as here presented, Navas et al. (2004) also demonstrated that the robustness of the skull is also related to compression force among *L. microcephalum*. Individuals with wider heads produced greater compression forces (about 25 N). Likewise, Vanhooydonck et al. (2011) and Baeckens et al. (2016) showed a strong positive relation between head size and bite force in *A. percivali* and *Trogonophis wiemanni*, respectively. According to these authors, bite performance also increased with head size in these species.

Besides individuals of the *L. microcephalum* species, our sample contained a unique *L. scutigerum* specimen that presented the narrowest skull (6.5 mm), and, according to our results, it showed the highest mean speed. Comparing inter-specifically, the morphological features of *L. scutigerum* supported the results of the locomotor performance analysis, and the differences in relation to *L. microcephalum*.

Gans (1978) highlighted that the slight lateral protrusion of the otic capsules is important for improving the excavation efficiency, here corroborated by the negative correlation between the width of skull and speed through regression analysis.

According to our morphological results based on a larger sample, the species *L. scutigerum* presented a narrower skull (maximum width situated at the occipital region covering the otic capsules) when compared to *L. microcephalum* which possesses a wider skull.

Future studies on compression force and bite performance are needed for these species. Although *L. microcephalum* exhibited low speed during digging in relation to *L. scutigerum*, according to Navas et al. (2004), Vanhooydonck et al. (2011), and Baeckens et al. (2016), it might be able to exert greater compression and bite forces. Even small variations in head width may present a large impact on burrowing compression force (Navas et al., 2004) and speed (Vanhooydonck et al., 2011). Thus, our present data on speed, associated with the data from Navas et al. (2004) on compression forces suggest a performance trade-off between compression forces and burrowing speeds in the shovel-headed *Leposternon* (i. e. individuals with narrow heads excavate rapidly but exert less push force, while individuals with robust heads excavate slowly but exert more push force). This relation may be associated with the ecology and geographic distribution differences among *L. microcephalum* (larger geographic distribution occupying different soil types) and *L. scutigerum* (limited distribution occupying a restricted soil type).

ACKNOWLEDGMENTS

We thank Marcos José de Mello Pinheiro from Fundação Técnico Educacional Souza Marques, for lending the live *L. scutigerum* specimen; Marcos Paulo Silva de Almeida from Hospital Universitário Pedro Ernesto, for managing the x-ray machine and acquiring the images and footages; and Dr. Concepción Azorit Casas from Universidad de Jaén, Nicolas Snow from Academia English International Jaén, and Dr. Jeanine Salles dos Santos from UERJ for reviewing the manuscript. We also thank Dr. Timothy Moulton from UERJ for English reviewing the manuscript.

Funding Statement: The Prociência Fellowship Program/UERJ, FAPERJ (L.S.L.Hohl: E-26/101.070/2013; O. Rocha-Barbosa: E-26/111.279/2014), CAPES/DS and CNPq (O. Rocha-Barbosa: 302162/2012-7) financially supported this work.

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APÊNDICE D – *Leposternon microcephalum* (Reptilia: Squamata: Amphisbaenia) as a model to reconstruct the “shovel” excavation pattern in the virtual ambiance.

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Abstract

There is a clear progression of the techniques used in studies of fossorial locomotion, more specifically in the amphisbaenid *Leposternon microcephalum*. Over the years, the locomotor behavior of this species has been described from different perspectives such as: visual observation, external body filming and videofluoroscopy. In each technique used, the descriptions of its excavation behavior have been gaining new details that help in understanding the lifestyle of this species in the underground environment. Here we generate a three-dimensional model of the anterior portion of the skeleton of *L. microcephalum*. Then it was animated using the Scientific Rotoscopy technique that consists of using images of X-ray footage to position the 3D skeleton and reproduce its movement, in this case the excavatory cycle. Simulating the digging behavior of *L. microcephalum* in the virtual environment using Scientific Rotoscopy made possible to increase the studies of its biomechanics with a more sophisticated resources. One novelty was to observe the effective participation of the ribs during excavatory movements, which was provided by the use of said methodology.

Keywords: 3D biomechanics, Amphisbaenia, Excavatory cycle

Introduction

Fossorial animals have different locomotor strategies to perform in four conditions: 1) displacement in the soil surface; 2) displacement through an existing gallery; 3) to continue the construction of a pre-existing gallery on the ground; and 4) the penetration of the soil to leave the surface, building a new gallery (Gans, 1960; 1978) that can be constructed by disaggregation and subsequent removal of the substrate (most mammals, birds and certain annelids and arthropods), and temporary ("sand-swarming" animals such as *Neoseps* lizards) or permanent compaction (Amphisbaenas and snakes of the Uropeltidae family) (Gans, 1960, 1969, 1976). A greater degree of difficulty is found in situations 3 and 4, where the Amphisbaenia have a recognized degree of specialization expressed by the speed and efficiency with which they build their galleries even in compacted soils (Gans, 1978).

Amphisbaenians are fossorial reptiles morphologically specialized for dig (Gans, 1969). The strongly ossified and compact skull, characteristic of the group, may assume four different morphological patterns: spade-head, keel-head, round-head, and shovel-head (Kearney, 2003). Those morphotypes are associated to different excavation patterns: random movements of the head (round-headed), lateral movements (keel-headed), “shovel” (shovel-headed), and “screw” (spade-headed) movements (Gans, 1974).

When amphisbaenians move on the surface of the ground, they can use the lateral ondulation of the body, also called serpentine locomotion, which may be associated with concertina movements when the surface is smooth (Gans, 1974), as well as using rectilinear locomotion (Gans, 1978). The latter is used for displacement at the surface of the soil by all representatives of the Trogonophidae family and by those with the most robust body within Amphisbaenidae. In order to move within the galleries, amphisbaenians may use rectilinear locomotion, concertina (Gans, 1978) and a variation of the latter called "internal concertina" (Gans, 1973) previously called "vermiform locomotion" by Gaymer (1971).

The genus *Leposternon* (Amphisbaenidae, South America) has the shovel-headed pattern (Gans, 1974, 2005; Kearney, 2003), thus associated to the “shovel” movements of the head when digging its galleries (Gans, 1974). *Leposternon*, considered the most specialized for digging (Gans, 1974, 2005; Kearney, 2003; Hohl et al, 2014), posses ten recognized species (Ribeiro, Santos-Jr & Zaher, 2015), and more recently, *L. mineiro* sp. nov. was described from Cerrado biome (Ribeiro, Silveira & Santos-Jr, 2018).

Among other Amphisbaenia species, *L. microcephalum* Wagler, 1824, has the greatest attention of studies on fossorial locomotion. According to Hohl et al. (2017), in the last 60 years, the locomotor behavior of the species has been studied through visual observations (Kaiser, 1955), motion pictures of the external body (Navas et al., 2004), and the videofluoroscopy technique (Barros-Filho et al., 2008; Hohl et al., 2014). The most recent description of the excavatory cycle of *L. microcephalum* is presented as: (1) initial static position with the gular and anterior body regions lying over the tunnel floor; (2) retreating and downward bending of the head, with tip of snout touching the floor substrate; (3) a continuous upward and forward head movement, which compacts the substrate granules against the tunnel roof, while the pectoral region compresses the tunnel floor. This is followed by the dropping of the head, returning to the initial static position (Barros-Filho et al., 2008; Hohl et al., 2014).

Regardless of the type of movements, these are performed by contractions of specific muscle groups that lead to move bone structures, leading to flexion, extension or rotation of parts of the body during a certain type of displacement. For example, among other muscles associated to the animal's body movement, the *Musculus longus colli* or *longus cervicis* is one of the four deep cervical flexor muscles together with *M. longus capitis*, *M. rectus capitis anterior* and *M. rectus capitis lateralis*. In Amphisbaenia, the Element X (wedged at the lateral margins of the basioccipital-basisphenoid suture) is the insertion point of this muscle that originates on the cervical vertebrae (Montero *et al.*, 2017). It acts with the other cervical flexors to produce neck flexion, retreating and downward the head.

The *M. longissimus dorsi*, a collection of three separate muscles (*M. longissimus capitis*, *M. longissimus* and *M. longissimus thoracis*), run up nearly the entire length of both sides of the spinal column. In *L. microcephalum* it is an extensive dorsal muscle that starts at the base of the head and runs backwards along the dorsal body of the animal (Navas et al., 2004). It is less exaggerated in amphisbaenians that use random head movements while digging, such as *Amphisbaena alba* (Gans, 1978). The *M. longissimus dorsi* is bilateral, with well definid right and left sides that are separated by a central tendon (Navas el al., 2004), and its function invloves to move both the back and the neck, including movements as neck extension, bending the neck backwards elevating the head.

The superior costo-cutaneous muscle originates in the dorsal extremity of the ribs and inserts on the ventral skin and the inferior costo-cutaneous muscle originates in the ventral extremity of the ribs with the same attachment. Toghether, they move the ribs and press the ventral scales on the substrate, moving the vertebral axis forward (Renous, 1994).

There is a clear progression of the techniques used in studies of fossorial locomotion, more specifically in *L. microcephalum*. In both techniques used, the descriptions of its excavation behavior have been gaining new details that help in understanding the lifestyle of this species in the underground environment. Thus, we hypothesize that *L. microcephalum* is a great model to simulate an excavatory behavior in the virtual environment (*in silico*), increasing the studies of its biomechanics through sophisticated resources. The objectives were to make a three-dimensional reconstruction of the skeleton of this species, and animate it in the virtual environment through the technique of Scientific Rotoscopy based on videofluoroscopy images.

Material and Methods

The Scientific Rotoscopy is a method of motion analysis that takes advantage of the quality of digital tools to create skeleton models and thus extract three-dimensional kinematic data from videofluoroscopy or conventional external body footage, and render animations (i. e. compile and obtain the final product of digital processing) accurately (Gatesy & Alenghat, 1999; Gatesy *et al.*, 2004, 2007).

According to Gatesy *et al.* (2010), the objective of Scientific Rotoscopy is to simultaneously animate and quantify the movement of an animal's skeleton, positioning a 3D computer model according to the perspectives of traditional external body filming or videofluoroscopy. This technique involves three main steps: 1) elements of the experimental setup are recreated as a 3D scene using animation software; 2) three-dimensional models of the bones are created from computed tomography and articulated by virtual articulations in a digital puppet (rigging); and 3) this skeletal marionette is represented by the alignment of each bone model to correspond to the background images generated from the videofluoroscopy (Gatesy *et al.*, 2010).

A videofluoroscopy record is a technique that has been used in behavioral studies of amphisbaenians (Barros-Filho et al., 2008; Hohl et al., 2014). The footages of an individual of *L. microcephalum* analyzed by Hohl *et al.* (2014) were used in the present study. According to the authors, the X-ray images were taken at the Pedro Ernesto University Hospital (HUPE / UERJ), Rio de Janeiro, through a videofluoroscopy machine Toshiba (Toshiba Ultimax of Toshiba Medical Systems) that shoots at 30 frames per second, calibrated in 200 μA and 40 kV.

A three-dimensional reconstruction of the skeleton of a fixed specimen of *L. microcephalum* (Herpetological Collection of the Universidade do Estado do Rio de Janeiro, UERJ 710) was carried out by computerized X-ray microtomography (µCT). The specimen was scanned in the Laboratório de Instrumentação Nuclear (LIN) of the Universidade Federal do Rio de Janeiro (UFRJ) using a microtograph model SkyScan 1173.

The X-ray source operated at 50 kV and 160 μA. The source-to-object distance was 115.42 mm. The data set consists of 2.055 high-resolution computed tomography (CT) slices taken along an oblique axis of the anterior portion of the animal's body from the tip of the snout to the 7th vertebra. Each image of the slices was pooled at a resolution of 2240 X 2240 pixels and represents a thickness and spacing of 15.67 μm. The three-dimensional reconstruction of the bones, "rendering", based on the slices generated was done in Mimics (Materialise, 2010). The files were exported as OBJ format known as Object File Wavefront 3D, and then converted in STL (Standard Tessellation Language), compatible with the softwrare where the skeleton animation was made.

The step of animation of the skeleton was done using the software Autodesk Maya version 2012 (Autodesk, Inc.), following the basic principles of the Scientific Rotoscopy technique described previously (Gatesy & Alenghat, 1999; Gatesy *et al.,* 2004, 2007).

Initially, an animation skeleton called “rigging” was generated, which is used to control the movements of the virtual model, making it possible to reconstruct the excavation pattern exhibited by *L. microcephalum*. This is composed of a set of joint chains that have a system of hierarchy of movement. Thus, the so-called “parent-joint” influences the movement of what are considered “child-joints”, which may also have independent movements (Loguercio, 2010; Chagas, 2016). In addition, the rigging also has the “root-joint” that is its first joint in the hierarchy, acting as the center of gravity (Chagas, 2016).

To create the rigging based on the real skeleton of the anterior portion of the *L. microcephalum* body, pivot points were generated respecting the real joints. They are: skull - atlas/axis; atlas/axis - vertebra 3 (vert.3); vert.3 - vert.4; vert.4 - vert.5; vert.5 - vert.6; vert. 6 - vert. 7; vert.7 - vert.8; rib1 - vert.4; rib2 - vert.5; rib3 - vert.6; rib4 - vert.7; rib5 - vert.8 (Fig. 1). From the kinematics feature, joint movements were determined by FK (forward kinematics), IK (inverse kinematics) and spline IK manipulators by clusters (Fig. 2).

Figure 1 – Referente à Figura 28 da presente Tese

Figure 2 – Referente à Figura 29 da presente Tese

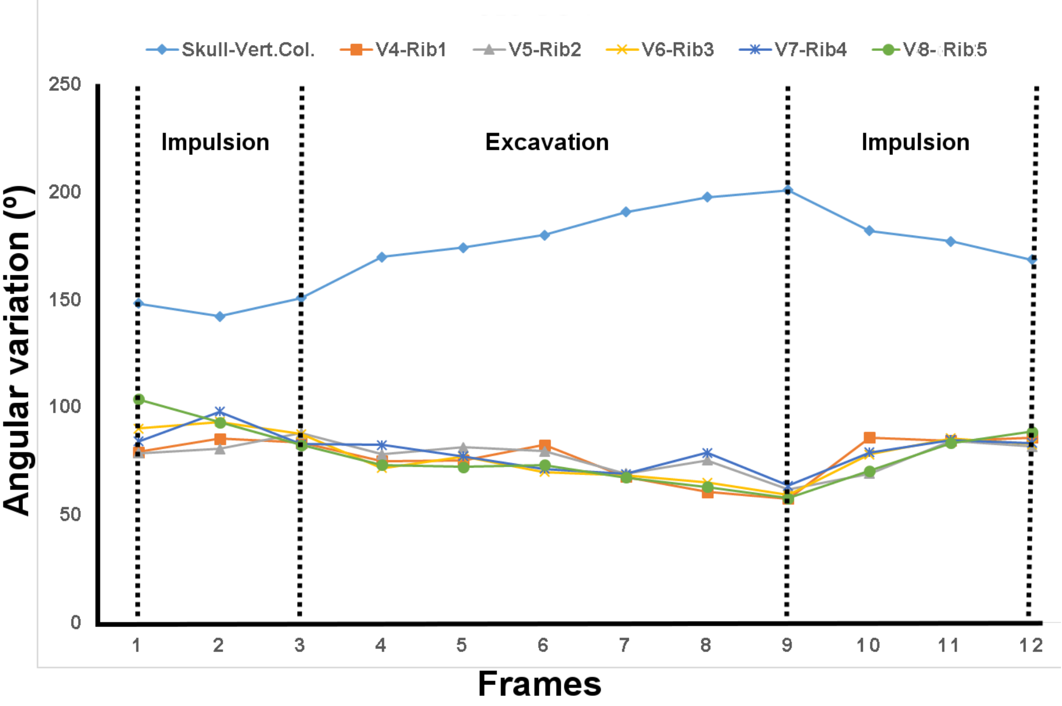
After the rigging ready, videofluoroscopy footage containing sequences from the excavation cycles performed by *L. microcephalum* was selected in order to capture the set of images that compose the execution of an excavation cycle. These were captured every three frames, totaling 16 images to complete an entire excavatory cycle. Then, the images were imported into the software Autodesk Maya and the rigging was positioned according to the X-ray images of the excavatory cycle movement, giving great confidence of its position in relation to the real animal skeleton. With the animated skeleton ready and positioned, this was associated with the three-dimensional model of the real skeleton, imported in STL format, which was then animated. Angular variation of the skull in relation to the vertebral column and the ribs in relation to the corresponding vertebra was determined to quantify the effective participation of the ribs in the excavatory movements.

According to information in the literature about the musculature involved in the process of locomotion movements of amphisbaenids, the muscle groups of the anterior region, possibly involved in the digging process, were represented and inserted manually on the three-dimensional skeleton of *L. microcephalum*. Thus, the muscular group of the ventral part, related to the flexion of the head (*Musculus* *longus colli*, *M. longus capitis*, *M. rectus capitis anterior*, and *M. rectus capitis lateralis* (Montero et al., 2017)), of the dorsal part related to head elevation [*M. longissimus dorsi* (Navas et al, 2004); and the group related to the movement of the ribs during the displacement [superior and inferior costo-cutaneous muscles (Renous, 1994)].

Results

A 3D model of the skeletal structures of the anterior body of *L. microcephalum* was produced. Then, the *in silico* representation of the excavatory cycle of this species was simulated and analyzed. In addition to the excavation pattern previously decribed, we observed the effective participation of the ribs that aid in the animal's displacement inside the gallery. While the individual put the head down (the angle between the skull and the spine closes) the ribs move subtly forward (the angle between the ribs and their respective vertebrae opens). In the same way, while the individual keep the head up (the angle between the skull and the spine opens) the ribs move subtly backward (the angle between the ribs and their respective vertebrae closes). The angular variation of these structures during the excavatory cycle is showed in the Figure 3.

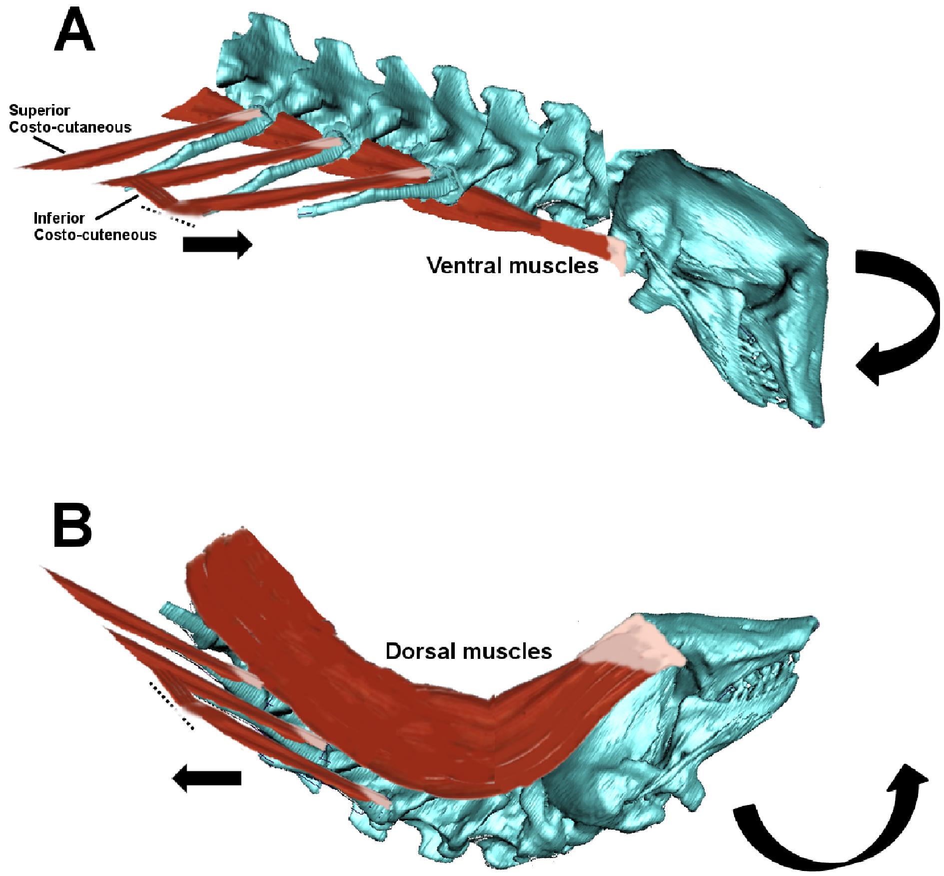
Figure 3 – Angular variation of the skull and ribs during a excavatory cycle.



Legend: Skull-Vert.Col = Angle between skull and vertebral column; V4-Rib1 = Angle between the first rib and the vertebrae 4; V5-Rib2 = Angle between the second rib and the vertebrae 5; V6-Rib3 = Angle between the third rib and the vertebrae 6; V7-Rib4 = Angle between the fourth rib and the vertebrae 7; V8-Rib5 = Angle between the fifth rib and the vertebrae 8. Impulsion = retreating and downward bending of the head; Excavation = continuous upward and forward head movement.

Based on the the most recent description of the excavatory cycle of *L. microcephalum* (Barros-Filho et al., 2008; Hohl et al., 2014) we presented some new observations in the steps observed by those authors: (1) initial static position with the gular and anterior body regions lying over the tunnel floor; (2) a group of ventral muscles (*M. longus colli*, *M. longus capitis*, *M. rectus capitis anterior*, and *M. rectus capitis lateralis*) contract and the head downward bending, thus the tip of snout touches the floor substrate. At the same time the costo-cutaneous muscles moves the ribs forward, opening the angulation in relation to the correspondent vertebrae, thus aiding in a subtle body retraction; (3) the dorsal muscles of *M. longissimus dorsi* complex contract moving the head upward while the costo-cutaneous muscles moves ribs backward, closing the angulation in relation to the correspondent vertebrae, aiding in a continuous forward body movement, thus compacting the substrate granules against the tunnel roof and floor. This is followed by the dropping of the head, returning to the initial static position (Figure 4). During the execution of the excavatory cyles the ribs of the anterior region of the body function like the oars of a Viking boat.

Figure 4 – Squeme showing the muscles involved in the retreating and downward bending of the head, and in the continuous upward and forward head movement during an excavatory cycle.



Legend: Short arrows indicate the movements of the ribs and the curved arrows indicate the movements of the head. Dotted lines represent the attachment of the costo-cutaneous muscles in the skin.

Discussion

According to Renous (1994) the costal gridle has considerable mechanical importance. It must, by its rigidity, ensure the positioning of the viscera while ensuring the respiratory movements. In the rectilinear locomotion, used to move within the galleries associated with other locomotory strategies (Gans, 1978), the whole body moves forward, straight, without any curvature. This mode of locomotion is the result of the action of the contraction waves of cost-cutaneous muscles, traversing the body from one end to the other (Renous, 1994). The contracion of the superior costo-cutaneous muscle determines the recovery of each ventral scale, which is supported on the substrate. The mobility results from the collapse of the scales, by relaxation of this last muscle and contraction of the inferior costo-cutaneous. The ventral side of the animal progresses therefore by successive stages. The rigid vertebral axis receives, by the play of the ribs and the musculature, the propulsive forcing resulting from the body wall, and advances in a continuous way, like a boat being paddled (Renous, 1994). Our results showed that during the excavatory movements of *L. microcephalum* the ribs follow the pattern described above and have an effective participation in the excavation process.

Scientific Rotoscopy is a morphology-based method for quantifying and visualizing vertebrate movement that offers distinct benefits (Gatesy *et al.*, 2010), thus a high-resolution, digital counterpart of aligning a physical skeletal to x-ray images of a moving animal (Jenkins & Goslow, 1983; Jenkins et al., 1988; Dial et al., 1991). This method deals directly with accurate 3-D representations of the morphological structures of interest, fully integrating morphology into motion analysis (Gatesy *et al.*, 2010). The present study innovate locomotor behavior studies on fossorial animals through Scientific Rotoscopy, wich use a bone’s entire x-ray shadow and its articulation with adjacent bones to reconstruct position and orientation.

According to Hohl et al. (2017), in the last 60 years, the locomotor behavior of *L. microcephalum* has been studied through different perpectives. Kaiser (1955) made some observations through visual observations only, Navas et al. (2004) used motion pictures of the external body, and both Barros-Filho et al. (2008) and Hohl et al. (2014) observed the excatory behavior using the videofluoroscopy technique. In such studies the authors describe the excavating behavior of the species in different ways, generating some discussion about which description most represents reality. For example, Kaiser (1955) described the tunnelling locomotor behaviour of *L. microcephalum* as two-step gait, but Gans (1960) raised doubts about its accuracy; Navas et al. (2004) added more details now considering a three-step gait; both Barros-Filho et al. (2008) and Hohl et al. (2014) improved characterization and description of the three-step gait. Barros-Filho et al. (2008) have already pointed out that the use of less advanced techniques in previous works is responsible for the divergences found in the descriptions of locomotor behavior in *L. microcephalum*. Here, these descriptions become even more complex and detailed with relevant novelty that was the effective participation of the movement of the ribs aiding in the construction of the galleries and the progression of the animal inside them.

Thus, it is clear how the reliability and complexity of the descriptions of the locomotor behavior of this species increases according to innovations of the techniques and methods used. This is a characteristic of science in the sense that the production of scientific knowledge is related to technological innovation and vice versa.

Acknowledgment

We thank the funding statement: The Prociência Fellowship Program/UERJ, FAPERJ (L.S.L.Hohl: E-26/101.070/2013; O. Rocha-Barbosa: E-26/111.279/2014), CAPES/DS and CNPq (O. Rocha-Barbosa: 302162/2012-7) for financially supported this work.

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