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Abstract

Brain endocasts obtained from computed tomography are now widely used in the field of comparative neuroanatomy. They provide an overview of the morphology of the brain and associated tissues located in the cranial cavity. Through anatomical comparisons between species, insights on the senses, the behavior, and the lifestyle can be gained. Although there are many studies dealing with mammal and bird endocasts, those performed on the brain endocasts of squamates are comparatively rare, thus limiting our understanding of their morphological variability and interpretations. Here, we provide the first comparative study of snake brain endocasts in order to bring new information about the morphology of these structures. Additionally, we test if the snake brain endocast encompasses a phylogenetic and/or an ecological signal. For this purpose, the digital endocasts of 45 snake specimens, including a wide diversity in terms of phylogeny and ecology, were digitized using computed tomography, and compared both qualitatively and quantitatively. Snake endocasts exhibit a great variability. The different methods performed from descriptive characters, linear measurements and the outline curves provided complementary information. All these methods have shown that the shape of the snake brain endocast contains, as in mammals and birds, a phylogenetic signal but also an ecological one. Although phylogenetically-related taxa share several similarities between each other, the brain endocast morphology reflects some notable ecological trends: e.g., 1) fossorial species possess both reduced optic tectum and pituitary gland; 2) both fossorial and marine species have cerebral hemispheres poorly developed laterally; 3) cerebral hemispheres and optic tectum are more developed in arboreal and terrestrial species.

Key words: computed tomography, brain endocast, snakes, morphometrics, ecological signal, squamates, sensory information.

Introduction

Computed Tomography (CT) allows the reconstruction of high-quality 3D models of both hard and soft tissues that can be used for different purposes, such as anatomical and biomechanical studies. It thus constitutes an important exploratory tool in biology and opens a range of new possible investigations (e.g., Boistel et al., 2011a; Carril et al., 2015).

Computed tomography is now widely used to visualize the endocranial space with the construction of digital endocasts that may reflect the morphology of the brain and associated tissues (e.g., Anderson et al., 2000; Macrini et al., 2007; Olori, 2010; Bienvenu et al., 2011; Smith & Clarke, 2012; Racicot & Colbert, 2013; Ahrens, 2014; Carril et al., 2015; Corfield et al., 2015; Danilo et al., 2015; Gonzales et al., 2015, Kawabe et al., 2015), the inner ear (e.g., Chapla et al., 2007; Georgi & Sipla, 2008; Walsh et al., 2009; Ekdale, 2010, 2011, 2013; Willis et al., 2013), the vascular system (e.g., Porter & Witmer, 2015), the cranial nerves (e.g., George & Holliday, 2013), and pneumatic sinuses (e.g., Bona et al., 2013). Endocasts are generated at the interface between the skeleton (typically bone or cartilage) and the soft tissues (or fluid) lying immediately near it (Balanoff et al., 2015). In the cranial cavity, the soft tissue forming the interface with the surrounding skeleton is not the brain but the superficial surface of the dural meninges, blood vessels and vasculature enveloping the brain (Walsh & Knoll, 2011). Thus, brain endocasts provide only an overview of the external morphology of the brain itself. They may reflect the relative size of the different regions of the brain and could provide some information about sensory abilities, as well as about the behavior and ecology of the species (Walsh & Knoll, 2011).

The degree to which the brain endocast reflects the morphology of the brain depends on the degree to which the brain fills the cranial cavity. This factor can vary widely between lineages (e.g., Jerison, 1973; Hopson, 1979; Witmer et al., 2008; George & Holliday, 2013) and over ontogeny (e.g., Macrini et al., 2007; Hurlburt et al., 2013). From different age

classes, the brain of the marsupial *Monodelphis domestica* fills between 67.8 and 86.6% of the endocranial volume (Macrini et al., 2007), whereas that of the smallest alligators occupies about 68% of the endocranial space, and about 32% in the largest alligators (Hurlburt et al., 2013). Mammals and birds, which are generally considered as highly encephalized taxa (large brains relative to body size, Balanoff et al., 2015), tend to have brains that nearly fill the cranial cavity, resulting in a strong correlation between the volume and morphology of the brain endocast and those of the brain (Balanoff et al., 2015). Thus, similarly as the brain morphology that may reflect the influence of ecological, behavioral and/or phylogenetic factors (e.g. Lefebvre et al., 2004; Walsh & Milner, 2011), the brain endocast of these taxa tends to have both a phylogenetic and ecologic signals (Lyras & Van Der Geer, 2003; Macrini et al., 2007; Carril et al., 2015; Corfield et al., 2015). Additionally, there is an increasing number of studies performed from brain endocasts of mammals and birds. These studies are generally performed to understand the relation between the mass of the brain and the volume of the cast, but also to consider the intraspecific variability, reflecting either ontogenetic variation, sexual dimorphism, or both (e.g., Macrini et al. 2007; Bienvenu et al., 2011; Kawabe et al., 2015), or finally focusing on the interspecific variability (e.g., Kawabe et al., 2013).

Among vertebrates other than mammals and birds, it is generally admitted that the brain does not entirely fill the cranial cavity (Balanoff et al., 2015). A commonly cited estimate considers that the brain occupies only 50% of the endocranial space (Hopson, 1979). However, this ratio is only based on the observation of one *Sphenodon* and one *Iguana* brain specimens (Hurlburt et al., 2013), and is probably far from representing a general pattern in non-endotherms. For example, it has been shown that the brain almost entirely fills the endocranial space in some extant chondrichthyans and teleosts (Northcutt, 2002; Balanoff et al., 2015). Within Squamata (lizards, snakes and amphisbaenians), a wide range of brain

versus endocranial cavity proportions were found (Kim & Evans, 2014). The lowest brain–endocranial volume ratio is found in *Gecko gecko* (0.35), whereas the false monitor lizard *Callopistes maculatus* exhibits a brain that nearly fills the endocranial cavity (0.97). Moreover, snakes and amphisbaenians are known to have a brain that fills most of the endocranial space (Starck, 1979; Nieuwenhuys et al., 1998), with a very narrow space between the brain and the cranial wall. The brain may thus fill the intracranial cavity in some squamates, indicating that brain endocasts within these species may reflect the external morphology of the brain with a certain degree of accuracy.

Computed tomography (i.e., magnetic resonance imaging (MRI) and X-ray absorption, as well as X-ray phase-contrast imaging techniques) has already been used on skulls of squamates for different purposes, such as the study of the brain (e.g., Anderson et al., 2000), the ear (e.g., Walsh et al., 2009; Boistel et al., 2011b; Christensen et al., 2012; Yi & Norell, 2015), and the skull morphology (e.g., Rowe et al., 1999; Bever et al., 2005; Rieppel & Maisano, 2007; Comeaux et al., 2010), the vascular patterns (e.g., Porter and Witmer, 2015), and the lacrimal system (e.g., Souza et al., 2015). But to date only a single study has focused on the brain endocast (Olori, 2010). In her study, Olori reconstructed and described the endocast of the burrowing snake *Uropeltis woodmasoni* and thus provided the first description of a snake brain endocast. However, as no comparative data are available within squamates, the results obtained cannot be discussed in detail. To date, there are several studies published about the brain itself or the central nervous system of squamates (e.g., Senn, 1966; Senn & Northcutt, 1973; Hoogland, 1982; Smeets et al., 1986; Martinez-Garcia et al., 1991; Reperant et al., 1992; Lanuza & Halpern, 1997; Nieuwenhuys et al., 1998; Atobe et al., 2004; Butler & Hodos, 2005; Powell & Leal, 2014) but the exact relationship between brain endocasts and brain morphology remains currently untested for squamates. In addition, data about the brain endocast morphology in this clade are insufficient to fully interpret this structure.

The present contribution proposes the first brain endocast comparative study in squamates. It will focus on snakes that are of particular interest since they show a great diversity in morphology, and occupy a wide range of ecologies with e.g., fossorial, aquatic, and arboreal species (e.g., Heatwole, 1999; Greene et al., 2000). Here, we propose to provide a quantitative anatomical description of the brain endocast of a wide sample of snake species using different morphometric approaches in order to: 1) bring new information about this structure, its general traits within snakes and the variation occurring; 2) test if, as in mammals and birds, the brain endocast of snakes reflects a phylogenetic and/or ecologic signal.

Material and Methods

Material

The material consists of the skull of 45 snake specimens (38 genera and 43 species; see Table 1); illustrating the diversity of snakes in both phylogenetic and ecological (i.e., habitat) perspectives (see Fig. 1). The dataset is divided into six fossorial, seven arboreal, thirteen terrestrial, nine semi-aquatic and ten marine species (Heatwole, 1999; Houssaye et al., 2013; Anthony Herrel, pers. com.). The semi-aquatic group encompasses species that spend most of their time in freshwater without contact with the sea. Three specimens of a single species, *Python regius*, were analyzed in order to evaluate the intraspecific variation.

Data acquisition

Microtomography was performed in order to non-destructively digitize the brain endocast of the specimens. The skull of the specimens studied were scanned: (1) at the University of Poitiers (France), Institut de Chimie des Milieux et Matériaux of Poitiers (IC2MP, Poitiers, France) using a X8050-16 Viscom model (resolution between 16.7 and 32.3 µm; reconstructions performed using Feldkamp algorithm with DigiCT software, version 1.15

[Digisens SA, France]); and (2) at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) using third generation synchrotron microtomography on beamlines ID 19 and BM5 (resolution between 5.0 and 14.9 μm; reconstructions performed using filtered back-projection algorithm with the ESRF PyHST software).

Image segmentation and visualization were performed using VGStudioMax 2.2 (Volume Graphics Inc., Heidelberg, Germany) at the Palaeontology Imaging Unit of the MNHN/UMR 7207 CR2P and Avizo 7.0 (VSG, Burlington MA, USA) at the UMR 7179 MECADEV. The segmentation tools of these software packages were used to select the endocranial space of the specimens thereby allowing separation of the skull from the endocranial space, and to reconstruct the brain endocast.

Institutional abbreviations

IC2MP, Institut de Chimie des Milieux et Matériaux, Poitiers, France; **ESRF**, European Synchrotron Radiation Facility, Grenoble, France ; **MCZ**, Museum for Comparative Zoology, Harvard University, U. S. A.; **MNHN**, Muséum National d'Histoire Naturelle, Paris, France ; **ZRC**, Zoological Reference Collections, National University of Singapore.

Measurements

For each specimen, 21 measurements were defined and taken to illustrate the whole 3D shape, volume and surface of the brain endocast (see Fig. 2B). All the measurements made on the brain endocast were measured point-to-point and obtained with the digital caliper of VGStudioMax 2.2 and the measuring tool of Avizo 7.0, both with accuracy of 0.01mm (see Supporting information S1 & S2). The print of the sutures between the different skull bones visible on the brain endocast surface were used to define homologous distances. The following list introduces the measurements taken on the brain endocast. The different parts of

the brain endocast are named with the same terms as those used for the brain itself (see Fig. 2A), following Butler & Hodos (2005); however the terms used here do not have a neurological significance and are not related to neural structures.

(a) *Length of the brain endocast* **(LE)**: distance between the anteriormost part of the olfactory bulbs still entirely surrounded by the frontal bone and the tip of the suture left by the contact of the supraoccipital with the two exoccipitals on the dorsal surface of the brain endocast;

(b) *Length of the olfactory bulbs* **(LOB)**: distance between the anteriormost part of the olfactory peduncles still entirely surrounded by the frontal bone and the fronto-parietal suture;

(c) *Length of the groove between the olfactory bulbs* **(LG)**: distance between the anteriormost end of the groove between the olfactory peduncles and the fronto-parietal suture;

(d) *Height of the main olfactory bulb* **(HOB)**: at the level of the anteriormost part of the main olfactory bulb still entirely surrounded by the frontal bone;

(e) *Height of the olfactory peduncle* **(HOP)**: at the level of the fronto-parietal suture;

(f) *Width of the olfactory peduncles* **(WOP)**: at the level of the fronto-parietal suture;

(g) *Length of the fissura interhemispherica* **(LFI)**: distance between the fronto-parietal suture and the virtual limit made by the groove between the cerebral hemispheres and the optic tectum;

(h) *Maximal width of the cerebral hemispheres* **(WCH)**;

(i) *Lateral expansion of the cerebral hemispheres* **(LCH)**: distance between the fronto-parietal suture and the posterior end of the lateral margin of the cerebral hemispheres;

(j) *Maximal height of the cerebral hemispheres* **(HCH)**;

(k) *Maximal width of the optic tectum* **(WOR)**;

(l) *Length of the optic tectum* **(LOR)**: distance between the virtual limit made by the groove separating the cerebral hemispheres of the optic tectum (see Fig. 2B) and the tip of the V-shaped suture between the parietal and the supraoccipital (see Fig. 2A);

(m) *Height of the optic tectum* **(HOR)**: distance between the dorsal surface of the optic tectum and the triple point formed by the suture between the parietal, prootic and basisphenoid (see Fig. 2A);

(n) *Length of the pituitary gland* **(LP)**: distance between the fronto-parietal suture and the most posterior point of the pituitary bulb;

(o) *Height of the pituitary gland* **(HP)**: distance between the most ventral point of the pituitary gland and the triple point formed by the sutures between the parietal, prootic and basisphenoid;

(p) *Width of the inner ear region* **(WIE)**: distance between the two triple points formed by the sutures of the supraoccipital, prootic and exoocipital;

(q) *Dorsal width of the posterior end of the brain endocast* **(DWPE)**: distance taken at the level of the suture between the supraoccipital and the two exoccipitals seen on the dorsal surface of the brain endocast;

(r) *Length of the posterior part of the brain endocast* **(LPE)**: distance between the tip of the V-shaped suture between the parietal and the supraoccipital, and the tip of the V-shaped suture between the supraoccipital and the two exoccipitals;

(s) *Height of the posterior part of the brain endocast* **(HPE)**: distance between the maximum of concavity of the inner ear region and the ventral margin of the brain endocast;

(t) *Width of the ventral part of the brain endocast* **(WPE)**: distance between the two triple points formed by the suture between the prootic, basisphenoid and basioccipital on the ventral margin;

(u) *Width in the pituitary gland region* **(WP)**: distance taken on the ventral surface of the brain endocast, between the triple points formed by the sutures between the parietal, prootic and basisphenoid.

Quantitative analyses

In order to provide complementary information, three different approaches were used to study the brain endocast variability occurring in snakes.

Descriptive character analysis

The differences observed between the various snake brain endocasts were listed and coded (See Supporting information S3: List of the characters and Matrix). We used the coded characters to run a principal coordinate analysis (PCoA) in order to evaluate the distances between the taxa and thus to identify which taxa are similar in brain endocast morphology based on these coded characters: the closest the species, the more similar the brain endocast morphologies.

Measure analysis

All data (see Supporting information S1 & S2) were log10-transformed prior to analysis to meet assumptions of normality and homoscedasticity required for parametric analyses. All the analyses were performed using the statistic software R (R Development Core Team, 2008). To analyze shape components independently from size, the log-shape ratios (Mosimann & James, 1979) were calculated based of the raw log10-transformed linear dimensions of the brain endocast.

In order to take into account the biases induced by measurement repeatability, three specimens of *Python regius* showing the lowest shape variation were selected. According to

the data published by Aubret et al. (2005), the comparison of their jaw length seems to differentiate a neonate specimen (P1; jaw length = 25.4 mm) from a juvenile (P3; jaw length = 31.4 mm) and an adult (P2; jaw length = 40.3 mm) ones. Ten repetitions were performed for each measure on these three specimens. Then, to quantify and visualize the differences between repetitions, a Principal Component Analysis (PCA) was performed. Shape differences between specimens were much higher than shape differences induced by repetitions (see Supporting information S2).

To evaluate the phylogenetic signal in the shape on the brain endocast in snakes, we used a multivariate generalization of the K statistic of Blomberg et al. (2003): the *K*mult (Adams, 2014). The phylogenetic signal is based on a phylogenetic consensus tree derived from several published phylogenies (Pyron et al., 2011; Hsiang et al., 2015; Lee & Scanlon, 2002; Fig. 1). Adams (2014) demonstrated that values of *K*mult <1 imply that taxa resemble each other phenotypically less than expected under Brownian motion whereas values of *K*mult >1 imply that close relatives are more similar to one another phenotypically than expected under Brownian motion. A PCA was also performed on the data obtained from the measurements made on the 45 snake specimens; the mean of the 10 measurements taken on each of the *Python regius* specimens was used.

To test the relationships between the habitat/ecology and the morphology of the brain endocast, the sampled taxa were classified into five habitat categories (see Fig.1): fossorial, terrestrial, arboreal, semi-aquatic, and marine (Heatwole, 1999; Houssaye et al., 2013; A. Herrel, pers. comm.). We performed a standard and phylogenetic MANOVA, to respectively evaluate whether the brain endocast variability could reflect the ecology, taking or not the phylogenetic relationships into consideration.

Outline curve analysis

For each brain endocast, the ventral and lateral views were selected to perform an outline curve analysis using geometric morphometrics (Zelditch et al., 2004). We used 2D sliding semi-landmarks (Gunz & Mitteroecker, 2013) that permit accurate description of homologous anatomical curves devoid of anatomical landmarks. Sliding semi-landmarks are allowed to slide, minimizing the bending energy between each specimen and the mean shape of the data set. This step creates a geometric homology between specimens that permits all classical geometric morphometric analyses. We performed a General Procrustes Superimposition to work on shape (Rohlf, & Slice, 1990) and PCAs for each view.

The dorsal view was not used here because of the difficulty to distinguish homologous outline curves on the posterior part of the structure at the level of the inner ear position. In ventral view, the 45 brain endocasts of our dataset were used. In lateral view, we used the posterior crest formed by the inner ear and three homologous points as landmarks to facilitate the placement of the curve semilandmarks. The sutures between the different skull bones visible on the posterior part of the brain endocast surface were used to define homologous points. The first point corresponds to the triple point formed by the sutures between the basioccipital, exoccipital and prootic. The second is the triple point formed by the prootic, the basioccipital and the basisphenoid. The last point represents the most ventral point of the suture between the basioccipital and the basisphenoid. In lateral view, we used 38 specimens because the sutures are not visible and did not allow the placement of the same landmarks on *Aipysurus eidouxii*, *Cerebrus rynchops*, *Corallus hortulanus*, *Dispholidus typus*, *Mimophis mahfalensis*, the smallest specimen of *Python regius* and *Uropeltis pulneyensis*.

Results

General description of snake endocast and variability

Here, only a description of the brain endocast will be provided, without considering the cranial nerves or the inner ear (data in Boistel et al., 2011b; Yi & Norell, 2015). The cast of the endocranial space does not only reflect the brain itself: associated tissues (e.g., venous system) are also reconstructed during segmentation and may hide some parts of the brain. The endocast morphology resulting from the segmentation of the endocranial space is described below as a whole. The brain endocast in snakes is surrounded dorsally by the frontal and parietal (anteriorly) and the supraoccipital and exoccipital (posteriorly), laterally by the prootics, and ventrally by the basioccipital and para-basisphenoid. The surface of the brain endocast of snakes is smooth.

Telencephalon

The telencephalon includes the olfactory bulbs, the olfactory peduncles and the cerebral hemispheres (see Fig. 2A). The main and accessory olfactory bulbs correspond to the anteriormost structure of the brain endocast (see Fig. 2A); however, from the brain endocast only it is not possible to distinguish one from another. They are attached to the rostral pole of the cerebral hemisphere by short olfactory peduncles. In dorsal view, a groove is visible running between the two olfactory bulbs. Posteriorly, the cerebral hemispheres represent the largest part of the brain endocast and gradually widen laterally. An interhemispheric fissure may be visible on the dorsal surface of the brain endocast, as attested by a groove between the cerebral hemispheres. The length of the interhemispheric fissure and the depth of the groove vary according to taxa.

Some taxa may exhibit olfactory bulbs wider than long, giving a short and stout aspect (e.g., width/length aspect ratio superior to one in *Typhlophys squamosus*, see Fig. 4A) in

dorsal view, while most taxa have an olfactory structure longer than wide (e.g., width/length aspect ratio inferior to one in *Hierophis viridiflavus*, see Fig. 4B). The lateral margin of this structure may be mediolaterally convex (e.g., *Acrochordus granulatus*, see Fig. 4D), relatively straight (e.g., *Eunectes murinus*, see Fig. 4E) or mediolaterally concave (e.g., *Hierophis viridiflavus*, see Fig. 4B) in dorsal view. Most species possess in dorsal view a system composed of two parallel olfactory bulbs and peduncles (e.g., *Eunectes murinus*, see Fig. 4E). Some others show a projection that diverges laterally from the fronto-parietal suture (e.g., *Homalopsis buccata*, see Fig. 4F), whereas others share the two conditions with parallel olfactory bulbs and peduncles diverging laterally at their anterior end (e.g., *Hierophis viridiflavus*, see Fig. 4B). In lateral view, the ventral margin may be ventrodorsally concave (e.g. *Mimophis mahfalensis*, see Fig. 5D), convex (e.g., *Boiga dendrophila*, see Fig. 5B) forming a bulge, or straight (e.g., *Homalopsis buccata*, see Fig. 5C). Some taxa (e.g., *Typhlophys squamosus*, see Fig. 4A) do not show any separation over the whole length of the olfactory peduncles in dorsal view. Most taxa have olfactory peduncles diverging only at their anterior end (e.g., *Hierophis viridiflavus*, see Fig. 4B). Some species have a large space between the two olfactory structures, separating them along almost their entire length (e.g., *Acrochordus granulatus*, see Fig. 4D). The width of the olfactory bulbs may vary antero-posteriorly. At the level of the fronto-parietal suture and in dorsal view, some taxa possess a posterior part as wide (e.g., *Eunectes murinus*, see Fig. 4E) or wider (e.g., *Cylindrophis ruffus*, see Fig. 4C) than the anterior end. However, others have olfactory bulbs with an anterior end wider than the posterior part (e.g., *Hierophis viridiflavus*, see Fig. 4B).

The relative size of the cerebral hemispheres varies between taxa. A distinction is seen between those that have hemispheres wider than long (e.g., width/length aspect ratio close to 1.4 in *Chrysopelea ornata*, see Fig. 4G) and those that have a structure as long as wide (e.g., width/length aspect ratio close to one in*Typhlophys squamosus*, see Fig. 4A). A

few taxa are exceptions with cerebral hemispheres longer than wide (e.g., width/length aspect ratio close to 0.3 in *Cylindrophis ruffus*, see Fig. 4C). The lateral extension in dorsal view generally begins just posterior to the fronto-parietal suture (e.g., *Eunectes murinus*, see Fig. 4E) but two taxa (*Cylindrophis ruffus* [Fig. 4C] and *Anilius scytale* [Fig. 4H]) exhibit cerebral hemispheres with an anterior part as wide as the fronto-parietal suture, the lateral extension occurring more posteriorly. In dorsal view, the lateral margin may be rounded (e.g., *Eunectes murinus*, see Fig. 4E) or relatively straight (e.g., *Chrysopelea ornata*, see Fig. 4G), providing a square appearance to the cerebral hemispheres. In lateral view, differences occur between taxa with cerebral hemispheres developed only along the horizontal axis (e.g., *Typhlophys squamosus*, see Fig. 5A), taxa with cerebral hemispheres developed in the horizontal plane but with a posterior part directed ventrally (e.g., *Homalopsis buccata*, see Fig. 5C) and taxa with a dorso-ventral extension at least as long as the horizontal one (e.g., *Boiga dendrophila*, see Fig. 5B). The limit between the cerebral hemispheres and the optic tectum depends on the lateral extension of the cerebral hemispheres. Species that do not have an important lateral extension (e.g., *Anilius scytale*, see Fig. 4H) do not show a clear delimitation between the optic tectum and the cerebrum, contrary to those that have a groove between the two structures and have laterally extended cerebral hemispheres (e.g., *Chrysopelea ornata*, see Fig. 4G).

Diencephalon

The pituitary gland, located ventrally to the cerebral hemispheres, is the only structure of the diencephalon seen on the brain endocast (see Fig. 2A); the pineal gland is not visible. In addition, the external morphology of the brain endocast does not allow the hypothalamus and the hypophysis to be delimited.

The pituitary gland may be marked by the presence in lateral view of a small bulge on the ventral surface of the brain endocast (e.g., *Anilius scytale*, see Fig. 5E). But generally the system shows a structure more developed ventrally, displaying (e.g., *Hierophis viridiflavus*, see Fig. 5F) or not (e.g., *Eunectes murinus*, see Fig. 5G) a posterior projection. Among those displaying a posterior projection, a distinction is made between those presenting a tilted system (e.g., *Enhydrina schistosa*, see Fig. 5H) and those having a posterior projection in the horizontal plane (e.g., *Hierophis viridiflavus*, see Fig. 5F). Differences relative to the ventral margin of the posterior projection also occur, between a curved (e.g., *Thamnophis sirtalis*, see Fig. 5J) and a flat (e.g., *Dispholidus typus*, see Fig. 5I) shape.

Mesencephalon

The mesencephalon lies posterior to the cerebral hemispheres. The optic tectum forms the roof of the mesencephalon (see Fig. 2A). From the endocast, the distinction between the optic tectum and the tegmentum, which is located more ventrally in the mesencephalon, is not possible. In dorsal view, the mesencephalon is less wide than the cerebral hemispheres.

In a few taxa this structure is not distinguishable from the cerebral hemispheres (e.g., *Typhlophys squamosus*, see Fig. 4A). In some others, the structure is visible in dorsal view only thanks to its decrease in width as compared to the cerebral hemispheres (e.g., *Eunectes murinus*, see Fig. 4E), and its surface appears smooth and flattened. However, in other species, the optic tectum exhibits in dorsal view a pair of domes separated by a median sulcus (e.g., *Thamnophis sirtalis*, see Fig. 6A). Some taxa show (in dorsal view) a distinct optic tectum as wide as the rhombencephalon (e.g. *Homalopsis buccata*, see Fig. 4F). The others have an optic tectum wider (e.g., *Chrysopelea ornata*, see Fig. 4G) or narrower (e.g., *Acrochordus granulatus*, see Fig. 4D) than the ventral margin of the rhombencephalon. In lateral view, most taxa possess a dorsal margin of the optic tectum located at the same height

as the cerebral hemispheres (e.g., *Eunectes murinus*, see Fig. 5G), except *Erpeton tentaculatum*, in which the margin is located more dorsally (see Fig. 6D).

Rhombencephalon

Posterior to the optic tectum, the cerebellum is not visible on the dorsal surface of the brain endocast. According to Aurboonyawat et al. (2008), the dorsal longitudinal vein located on the mid-dorsal surface of the brain endocast must cover it. On the lateral sides of the brain endocast, the large and round impressions indicate the position of the inner ear (see Fig. 2A). The *medulla oblongata* is located ventral to the inner ear region, and represents the ventral margin of the posterior part of the brain endocast.

Most species exhibit a rhombencephalon in lateral view with a rounded (e.g., *Boa constrictor*, see Fig. 7A) or straight (e.g., *Erpeton tentaculatum*, see Fig. 6D) ventral margin, but in some taxa (e.g., *Crotalus atrox*, see Fig. 7B) the ventral margin is triangular, pointing ventrally. The ventral extension of the *rhombencephalon* may correspond to the most ventral surface of the brain endocast in lateral view (e.g., *Boa constrictor*, see Fig. 7A) or not (e.g., *Dispholidus typus*, see Fig. 5I).

Quantitative analyses

Brain endocasts of snakes show a great variability. This variability is characterized by different relative proportions between the structures visible on the brain endocasts (e.g., size of the optic tectum compared to that of the cerebral hemispheres), giving a wide range of shapes, from stout (e.g., *Typhlophis squamosus*), to elongated and gracile (e.g., *Pelamis platurus*) or elongated and wide (e.g., *Boa constrictor*) brain endocasts. Below, this variability is analyzed quantitatively.

Descriptive character analysis

The results obtained (Fig. 8) show that 50.3 % of the variance is explained by the two main principal components (29.4 % and 20.9 % respectively). The distribution of the taxa indicates that fossorial and marine snakes are both distinct from those with other ecologies. Among the fossorial species, *Atractaspis irregularis* is quite distinct from two groups: the first one including *Uropeltis pulneyensis*, *Cylindrophis ruffus*, and *Anilius scytale*, and the second one made by *Typhlophis squamosus* and *Rhinotyphlops schlegelii*. *Micrurus lemniscatus* and *Acrochordus granulatus*, a terrestrial and a semi-aquatic snakes, respectively, tend toward the brain endocast morphology found in the fossorial taxa. Among the marine species of our dataset, *Enhydrina schistosa* and *Microcephalophis gracilis* are close to each other and distinct from other marine snakes. The terrestrial species show a wide distribution. The isolated position of *M. lemniscatus* was already cited above. *Hierophis gemonensis*, *Hierophis viridiflavus*, and *Thamnophis sirtalis* are close together and located near the two arboreal snakes *Dispholidus typus* and *Chrysopelea ornata*. These species are distinct from *Mimophis mahfalensis*, *Crotalus atrox*, *Agkistrodon contortrix*, *Coronella austriaca* and *Naja nivea*, which are close together and possess a brain endocast morphology similar to the arboreal snakes *Boiga dendrophila* and *Dasypeltis* sp. In addition, the three specimens of *Python regius* and *Candoia* sp., are distinct from the other terrestrial taxa with a brain endocast morphology tending towards those found in marine ones. Among the arboreal taxa not cited above, *Corallus hortulanus*, *Boa constrictor* and *Pareas margaritophorus* are close to the semi-aquatic snake *Cantoria violacea*. The distribution of the semi-aquatic species overlaps those of the terrestrial and arboreal snakes. The brain endocast of *Enhydris enhydris* is similar to that of *M. mahfalensis* and distinct from those of *Erpeton tentaculum*, *Fordonia leucobalia*, *Homalopsis buccata*, and *Enhydris punctata*, which are grouped together. The two

species *Cerberus rynchops* and *Eunectes murinus* are respectively close to *Candoia* sp. and to the three specimens of *P. regius*, and tend towards the marine taxa.

The species distribution suggests the existence of phylogenetic and ecological signals. Phylogenetically close species show more similarities than with other species (e.g., *Typhlophis squamosus* and *Rhinotyphlops schlegelii*). However, an ecological signal is also perceived, meaning that species sharing the same ecology show more brain endocast similarities than species with a different ecology.

Measure analysis

Intraspecific variability in *Python regius*

The PCA (see Fig. 9) shows that the two main axes explain 93 % of the variance (80 % and 13 % respectively). The repeatability test is positive as the ten iterations for each specimen are clearly grouped and the three specimens clearly distinct, indicating that the variability caused by the measurement acquisition is inferior to the variability between the specimens. All variables seem to act on the distribution of the specimens (though the impact of LP (Length of the pituitary bulb) on the second axis appears significantly more important than that of the other variables). The first principal component mostly separates the specimens based on size. The variables principally acting on PCA1 are the height of the olfactory bulbs (HOB), the length of the optic tectum (LOR) and the length of the pituitary gland (LP). The smaller specimen (P1) has the greatest height of the olfactory bulbs, the greatest length of the pituitary bulb, and the smallest length of the optic tectum. The second principal component separates the intermediate specimen (P3) from the two others. The main variable acting along the second axis is still the length of the pituitary gland (LP). The intermediate specimen (P3) shows the smallest height of the olfactory bulb, the greatest length of the optic tectum and an intermediate value for the length of the pituitary gland. Finally, the largest specimen (P2)

possesses the greatest length of the pituitary gland, and intermediate values for the height of the olfactory bulb and for the length of the optic tectum.

Interspecific variability

The PCA obtained with all snake specimens (see Fig. 10) shows that 60 % of the variance is explained by the two first axes (44.7% and 15.3% respectively). Fossorial species are clearly distinct from the others, with a great distribution along the first axis, contrary to the snakes with other ecologies, that all display a more limited distribution. The PCA shows some overlap between the snakes with arboreal, terrestrial, semi-aquatic and marine habitats, but a gradation is clearly visible. The arboreal and terrestrial taxa appear distinct (with no overlap) from the marine ones. All variables seem to act on the repartition of the species (see Supporting information S4). However, along the first axis, two variables mostly act on the distribution of the taxa: the width at the optic tectum level (WOR) and the dorsal width of the posterior end of the brain endocast (DWPE). The first axis seems to separate species that have an optic tectum as wide as the posterior end of the brain endocast (e.g., *Typhlophis squamosus*) from the ones in which the optic tectum is much wider than the posterior end of the brain endocast (e.g., *Pelamis platurus*). Along the second axis, the width of the olfactory peduncles (WOP) and the width of the cerebral hemispheres (WCH) explain most of the variability. These variables allow to distinguish species presenting a large difference between the width of the olfactory peduncles and the width of the cerebral hemispheres (e.g., *Boa constrictor*), from those that have a smaller difference between these two widths (e.g., *Cylindrophis ruffus*).

The MANOVA performed on the data indicates significant differences between brain endocasts depending on ecology (MANOVA: Wilks $\lambda = 0.751$, $F_{2, 22} = 8.75$, $P = 0.013$). The Kmult test indicates that brain endocast shape in snakes exhibits a significant phylogenetic

signal (Kmult = 0.814; P.value = 0.001), showing the importance to consider the phylogeny in studies of snake brain endocasts. The phylogenetic MANOVA still indicates significant differences pending on ecology (phylogenetic MANOVA: Wilks $\lambda = 0.0074$, $F_{2, 22} = 81.748$, Pphyl = 0.0087).

Outline curve analysis

The results obtained by the outline curve analyses (Fig. 11 and 12) enable to comment on the shape of snake brain endocasts according to the different ecologies.

The first PCA is obtained from the endocast outline curves in ventral view (Fig. 11) and shows that 61.9% of total variance is explained by the two first axes (44.6% and 17.3% respectively). The first axis separates proportionally stout brain endocasts, wide at the level of the olfactory bulbs and of the cerebral hemispheres (blue dotted line, Fig. 11, Axis 1), from longer and narrower endocasts (black dotted line, Fig. 11, Axis 1). Thus, brain endocasts of semi-aquatic, arboreal and terrestrial snakes are mostly wide, whereas the fossorial and marine species have an extended distribution along this first axis, encompassing both wide and narrow endocasts. However, the distribution of marine taxa is mainly concentrated towards narrow endocasts and only two species, *Aipysurus duboisii* and *Aipysurus eydouxii*, move towards wide endocasts. Along the second axis, the shape of the forebrain (olfactory bulbs and cerebral hemispheres) principally drives the distribution. Brain endocasts with wide olfactory bulbs have cerebral hemispheres located more anteriorly (dark dotted line, Fig. 11, Axis 2) than those with thinner olfactory bulbs (blue dotted line, Fig. 11, Axis 2). Semi-aquatic, fossorial and marine species all exhibit a brain endocast with wide olfactory bulbs and anteriorly located cerebral hemispheres, contrary to the arboreal and terrestrial snakes that are distributed all along the axis and thus express the two conditions.

The second PCA is obtained from the endocast outline curves in lateral view (Fig. 12) and shows that 65.8% of total variance is explained by the two first axes (48% and 17.8% respectively). The first axis illustrates brain endocasts with well dorsoventrally developed and ventrally oriented olfactory bulbs, and a posterior part characterized by a rounded dorsal surface more developed dorsally than the anterior part (blue dotted line in Fig. 12, Axis 1). These brain endocasts differ from those in which the olfactory bulbs are less developed dorsoventrally and dorsally oriented, and the posterior part presents a flat dorsal surface located at the same level as the anterior part (dark dotted line Fig. 12, Axis 1). The brain endocast of the fossorial species *Rhinotyphlops schlegelii* is well distinct from those of other taxa, with a structure very developed dorsoventrally and the posterior region higher than the anterior one. Arboreal and terrestrial species may show a mix between the two morphologies, with a well dorsoventrally developed brain endocast but a flat posterior region located at the same level as the anterior one. Marine snakes tend to have a flat brain endocast, whereas semi-aquatic and fossorial taxa show a large distribution presenting the two brain endocast morphologies. The second axis separates stout brain endocasts well developed dorsoventrally, with a slight dorsal constriction at the limit between the olfactory bulbs and the cerebral hemispheres (blue dotted line Fig. 12, Axis 2) from longer but less dorsoventrally developed brain endocasts (dark dotted line Fig. 12, Axis 2), with a ventral constriction at the limit between the olfactory bulbs and the cerebral hemispheres. The distribution of the taxa seems to indicate that the two morphologies are variably found in all ecologies. However, the dorsoventrally compressed brain endocast found in both marine (*Pelamis platurus*) and terrestrial (*Candoia* sp.) snakes, differs from the more dorsoventrally developed brain endocasts found in other taxa sharing their ecologies.

Discussion

Phylogenetic signal

We detected a significant phylogenetic signal in the snake brain endocast variability, meaning that it is at least partly constrained by shared ancestry. Indeed, some patterns or main trends in the brain endocast morphology reflect snakes' systematics. The scolecophidian snakes (*R. schlegelii* and *T. squamosus*, see Fig. 1) are the only ones presenting a brain endocast where the optic tectum is not visible (see Fig. 4A). Within the Booidae (see Fig. 1), the surface of the optic roof is smooth (e.g., *Eunectes murinus*, see Fig. 4E), and the pituitary gland is only developed ventrally. The Hydrophiidae (see Fig. 1) have cerebral hemispheres poorly developed laterally (e.g., *Enhydrina schistosa*, see Fig. 5H), contrary to the Colubridae (see Fig. 1) that possess cerebral hemispheres very developed both laterally and ventrally (e.g., *Hierophis viridiflavus*, see Fig. 4B, 5F), as well as an optic roof clearly visible with two distinct domes, and the olfactory bulbs widening on their anterior part. As the multivariate K was lower than one, species resemble each other less than expected under a Brownian motion model of evolution, which shows that, though significant, the phylogenetic signal remains weak. This suggests that other factors, such as ecology, do affect the snake endocast morphology.

Ecological signal

We also detected an ecological signal in the brain endocast of snakes, even when the phylogenetic relationships were taken into account. Though the different ecologies tested here are thus associated with morphological trends of the brain endocast, it nevertheless appears difficult to associate one structure with one ecology. Both standard and phylogenetic MANOVAs indicate significant differences between the ecologies, with an impact of all variables on the distribution of snakes. Thus, fossorial species have a brain endocast with a

poor lateral development of the cerebral hemispheres, and not visible or absent optic tectum and pituitary gland. Marine species exhibit an endocast more elongated, with cerebral hemispheres poorly developed laterally and projected only in the antero-posterior plan, but the optic tectum is clearly visible and the pituitary gland is developed ventrally. Brain endocasts of terrestrial and arboreal snakes differ from marine ones' by the great lateral extension of the cerebral hemispheres. Finally, it appears difficult to distinguish a common pattern for semi-aquatic snakes.

Within the same ecology, a great variability in brain endocast morphology can be observed. The results obtained from the outline curve analysis (Fig. 11 and 12) provide some examples. The cerebral hemispheres of *Aipysurus duboisii* and *Aipysurus eydouxii* are wider than long and developed ventrally on their posterior part, whereas in the other marine taxa of our dataset, the cerebral hemispheres are as long as wide and only directed in the horizontal plane. The brain endocasts of *Pelamis platurus* and *Candoia* sp. appear more flattened than those respectively found in other marine and terrestrial species. Finally, the morphology, the proportions and the orientation of the brain endocast of *Rhinotyphlops schlegelii* appear very distinct from those found in other fossorial snakes. It appears difficult to interpret these differences. It has been demonstrated that constraints imposed by the environment (e.g., habitat) and activity pattern have an impact in snake head shape, irrespective of the phylogenetic relationships (Fabre et al., 2016; Segall et al., 2016). These ecological constraints affect the brain endocast morphology in snakes as well. However, it is difficult to determine with certainty which ecological parameters mostly affect the brain endocast morphology. The two marine species, *A. duboisii* and *A. eydouxii*, have a brain endocast quite different from other marine taxa. It is unclear if these differences are related to changes in their skull morphology due to the fish-egg dietary specialization (Sanders et al., 2012) or if the particular morphology of their cerebral hemispheres has a sensory meaning. Similarly, the

flattened brain endocast of *Pelamis platurus*, not found in any other marine specimen from our dataset, could be related to modifications in the skull morphology associated with its pelagic condition, only known in this species, or to its unique foraging strategy at the oceanic surface through labile features such as slicks or drift lines (Brischoux & Lillywhite, 2011). It will be interesting to decompose the ecology in different factors (e.g., locomotion, prey capture mode) to determine which parameters mostly influence the snake brain endocast morphology.

Sensory inferences

Studies in mammals and birds have shown that the endocast morphology, like the brain morphology, may give some information about species sensory abilities (Sakai et al., 2011a,b; Corfield et al., 2012, 2015; Carril et al., 2015). Several studies on snake brain have shown a link between structure and function (e.g., Kubie et al., 1978; Halpern & Frumin, 1979; Halpern & Kubie, 1979; Friedman & Crews, 1985; Krohmer & Crews, 1987; Crews et al., 1988; Miller & Gutzke, 1999; Wyneken, 2007; Krohmer et al., 2010) but the link between sensory abilities and brain endocasts has never been investigated in snakes. According to Starck (1979) and Nieuwenhuys et al. (1998), the brain of snakes could fill the majority of the endocranial space, and thus reflect the brain anatomy. If it is the case, brain endocasts could provide information about their sensory abilities. The relationships between the brain and the brain endocast is currently untested in snakes (Olori, 2010), and was not the goal of this study.

In snakes, the main olfactory bulb (MOB) is responsible for capturing smells at the level of the olfactory epithelium, and transmitting them to the olfactory bulb; the accessory olfactory bulb (AOB) is responsible for pheromone processing related to chemical social communication and prey capture (Bales, 2014). The MOB projects mainly to the lateral cortex and the AOB mainly to the *nucleus sphericus* (Lanuza & Halpern, 1997), two structures

localized in the cerebral hemispheres. The MOB and AOB are involved in different behavioral activities, such as predation, mating and courtship (Bales, 2014). It is difficult to clearly identify the two structures and their limits from the brain endocast. However, morphological differences are perceived between the sampled taxa and they may imply differences in their sensory abilities. All snakes have a very developed vomeronasal system (Kubie & Halpern, 1979; Bales, 2014); however in hydrophiinae sea snakes the main olfactory bulbs are considered to be functionless and it seems that they use the AOB for smelling underwater (Schwenk, 2008; Schichida et al., 2013). Brain endocasts of hydrophiidae are indeed the only ones to show olfactory bulbs with a width increasing along the antero-posterior axis (e.g., *Enhydrina schistosa*, see Fig. 5H), which could correspond to a reduced MOB and a more developed AOB.

The cerebral hemispheres of snakes are composed of different structures (e.g., cortex, nucleus sphericus, anterior ventricular ridge, amygdala), each being considered as a link between the sources of sensory information and the brain structures that control and modulate the behavior (Halpern, 1980; Bales, 2014). Different studies about the lizard brain have shown that the medial dorsal cortices are relatively bigger in active foragers (Day et al., 1999a,b; 2001; Ladage et al., 2009). In snakes, males, which have a larger average territory than females, possess a significantly larger medial cortex than females (Roth et al., 2006). However, all these internal structures are not distinguishable on endocasts. Moreover, no comparative studies on snake brain endocasts have been performed to correlate size variation of these inner neural structures with endocast morphology. It is thus difficult to evaluate whether the different morphologies exhibited by the cerebral hemispheres of snakes involve differences in their sensory abilities.

The optic tectum in snakes is involved in the production of natural orienting movements in response to somatosensory, visual, and auditory stimuli (Nieuwenhuys et al.,

1998; Wyneken, 2007), and to signals from the infrared sensory system found in some snake families (Boidae, Pythonidae, and Crotalinae) (Goris, 2011). Several authors have shown that the size of the optic tectum is correlated to some behavioral traits and ecologies (Masai, 1973; Nieuwenhuys et al., 1998). For instance diurnal species have a larger optic tectum than burrowing species. From snake endocasts, it actually appears that all fossorial species have a reduced optic tectum, (e.g., *Cylidrophis ruffus*, see Fig. 4C), contrary to terrestrial and arboreal taxa, which have a large optic tectum (e.g., *Chrysopelea ornata*, see Fig. 4G). According to Lillywhite (2014), vision is better developed in arboreal snakes, and poorly developed in burrowing species and some aquatic species living in turbid waters. It seems thus possible to connect the size of the optic tectum to the development of vision. According to Masai (1973), the optic tectum of diurnal snakes is, as a rule, larger than that of nocturnal ones. However, the correlation between large optic tectum and diurnal activity is not clear. Some exceptions exist: the endocast of *Boiga dendrophila* (see Fig. 5B), a nocturnal snake (Rodda et al., 1999; Shivik et al., 2000), also shows a large optic tectum. There seems also to be no correlation between the occurrence of an infrared sensory system and the size of the optic tectum on endocasts. Specimens that have infrared organs (e.g., *Crotalus atrox*, see Fig. 7B) do not exhibit a larger optic tectum than specimens without infrared organs (e.g., *Boa constrictor*, see Fig. 7A). There is however one exception: *Erpeton tentaculatum* (see Fig. 6B-D), the only specimen which has an endocast with the dorsal margin of the optic tectum located more dorsally than the dorsal margin of the cerebral hemispheres. Such features can be correlated to the special nature of *E. tentaculatum*, which is the only snake presenting a pair of appendages that protrude from the face (Catania, 2011; 2012). The tentacles, useful to detect and locate preys, are innervated by trigeminal fibers to the optic tectum and could be responsible for its large size in *E. tentaculatum*.

Snake endocasts also show a great variability in the pituitary gland. This structure is generally considered to be structurally and functionally the most complex organ of the endocrine system (Harris & Donovan, 1966). Among vertebrates, the pituitary of snakes possesses some unique features: an asymmetrical structure flattened dorsoventrally and a pars tuberalis never developed (Schreibman, 1986). From the observation of brain endocasts only, a large variability is observed. However, it is not possible to determine whether this variability has a sensory significance. For example, brain endocasts of fossorial specimens have a clearly reduced pituitary gland but it is not clear whether this morphology is an adaptation reflecting the specialization of the skull due to fossorial activity (Rieppel, 1979; Rieppel & Zaher, 2000) or if this morphology has a sensory implication.

It is tempting to interpret the brain endocast variability in snakes through differences in sensory abilities between species; however, it is necessary to be very careful in the sensory inferences brought by an endocast study, which gives only an overview of the external morphology of the brain, and the complexity of the structure(s) must be taken into account.

Perspectives

The rapidly expanding interest in, and availability of, digital tomography data to visualize casts of the vertebrate endocranial cavity housing the brain (endocasts) represent new opportunities and challenges to the field of comparative neuroanatomy (Balanoff et al., 2015). In snakes, the brain endocast is still poorly known and the information associated with this structure remains untested. The different approaches used here have shown that snake brain endocasts contain both phylogenetic and ecological signals. However, the degree of influence of these two signals on the brain endocast morphology is difficult to interpret. It will be interesting to dissociate the variability due to each signal. Moreover, to fully understand the brain endocast structure and its variability among snakes, it appears necessary to

decompose the ecology in different parameters (e.g., locomotion, prey capture mode) in order to test whether one is particularly associated to one brain endocast structure.

Beyond the methodological approaches that we used in this study, the resort to three-dimensional geometric morphometrics (3DGM) would be interesting to improve the amount of shape changes taken into consideration. However, the difficulty of finding homologous anatomical landmarks would impose the use of sliding semi landmarks on surfaces (Gunz & Mitteroecker, 2013).

Cranial endocasts also represent a potentially large amount of unexplored phylogenetic data. Most morphological data for phylogenetic analyses of vertebrates come from the exterior shape of the skull (e.g., Gauthier et al., 2012). Internal cranial morphology is poorly represented in phylogenetic analyses because of the difficulty in visualizing and studying this anatomy. The advent of CT technology provides the potential to incorporate these new data into phylogenetic analyses.

Finally, in the context of the strong debate about the phylogenetic and ecological origin of snakes (e.g., Lee et al., 1999; Conrad, 2008; Hsiang et al., 2015; Martill et al., 2015; Reeder et al., 2015; Yi and Norell, 2015), endocranial studies might be of strong interest. Their application on crown snakes and lineages closely related to snakes (i.e., varanids, dibamids, mosasauroids) would provide major complementary information.

Conclusion

We used different methods to describe the brain endocast of snakes: descriptive characters, outline curve analysis, measurement series, and we observed a great variability in the brain endocast morphology of snakes. These methods provided different complementary information but all have shown that the shape of this structure contains, as in mammals and birds, a phylogenetic signal but also an ecological one. The different trends observed in the

brain endocast morphology distinguish the different ecologies, notably fossorial and marine snakes. The great diversity observed in the brain endocast of snakes, even within the same ecology, appears difficult to interpret and further analyses on the relation between brain endocast and ecological and sensory factors will be required. Biological inferences based on this structure should thus be made with caution and it is important to understand the complexity of this structure in order to avoid quick potentially wrong assumptions.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Appendix S1. Table of measurements taken on snake endocasts

Appendix S2. Table of measurements taken on the three *Python regius* specimens

Appendix S3. List of characters and matrix used for the PCoA

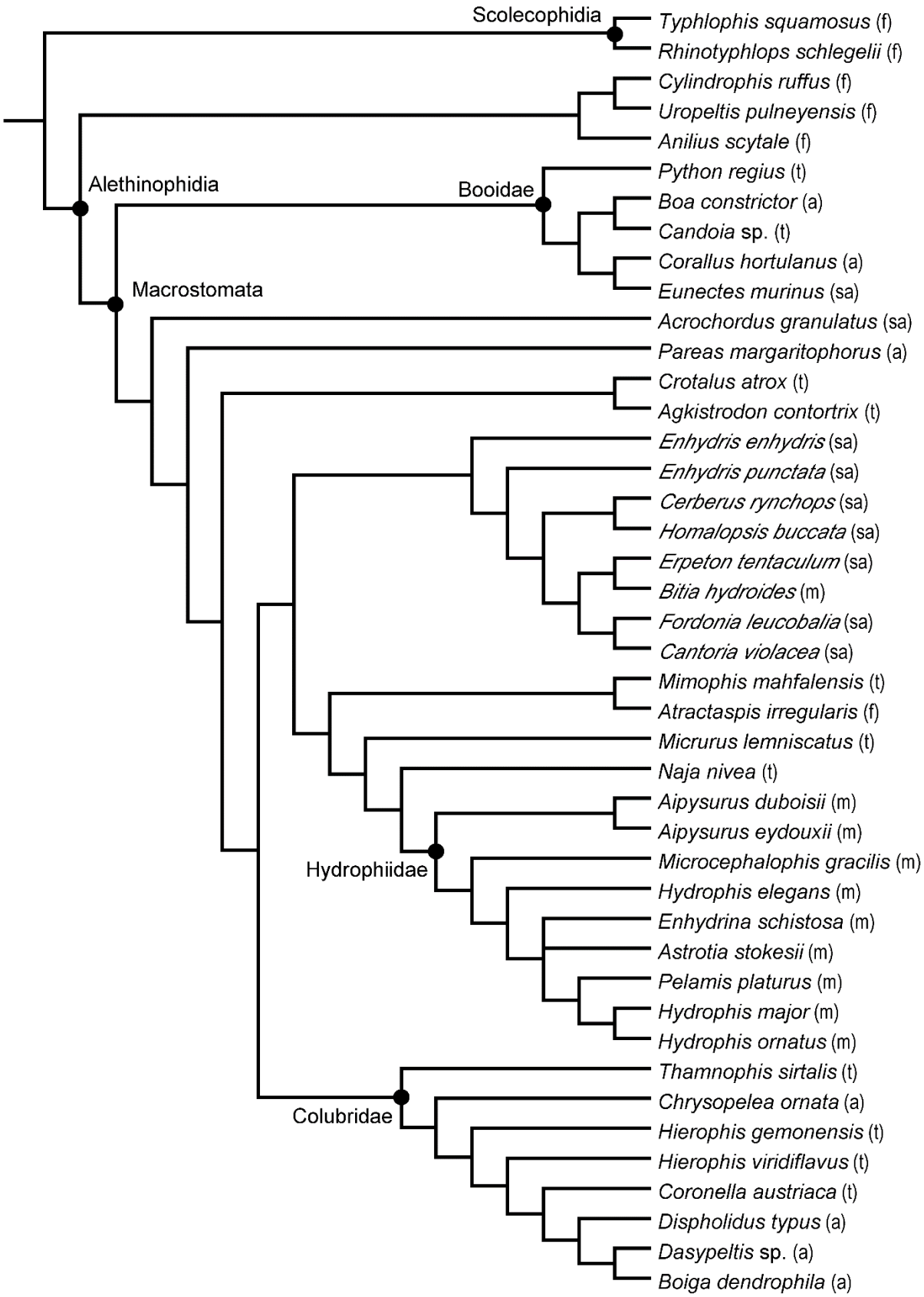
Appendix S4. Distribution of the variables in the principal component analyses performed on the 45 snake specimens.

Tables

Table 1. List of the material analyzed. Ha represents the categories based on habitat: A, arboreal; F, fossorial; M, marine; T, terrestrial; SA, semi-aquatic. AH, Anthony Herrel personal collections; GD, Gheylen Daghfous personal collections.

Family	Taxon	Ab.	Ha	Collection reference	Voxel size
Boidae	*Rhinotyphlops schlegelii*	Rs	F	AH Unnumb	13.3
Anomalepididae	*Typhlophis squamosus*	Ts	F	MNHN 1997.2042	5.1
Uropeltidae	*Uropeltis pulneyensis*	Up	F	MNHN 1994.0753	5.0
Cylindrophiidae	*Cylindrophis ruffus*	Cy	F	MNHN 1998.0201	20.1
Aniliidae	*Anilius scytale*	An	F	MNHN 1997.2106	10.1
Pythonidae	*Python regius*	P3	T	AH Unnumb	33.3
	Python regius	P2	T	AH Unnumb	28.9
	Python regius	P1	T	AH MS 37	21.6
Boidae	*Boa constrictor*	Bc	A	MNHN 1989.0177	7.6
	Candoia sp.	Cd	T	AH Unnumb	33.3
	Corallus hortulanus	Ch	A	AH MS 62	32
	Eunectes murinus	Em	SA	MNHN 1996.7898	7.6
Acrochordidae	*Acrochordus granulatus*	Ag	SA	ZRC 2.2334	24.2
Pareatidae	*Pareas margaritophorus*	Pm	A	MNHN 1974.1469	7.5
Viperidae	*Crotalus atrox*	Cr	T	AH MS 31	28.5
	Agkistrodon contortrix	Ac	T	AH MS 56	23.4
Homalopsidae	*Enhydris enhydris*	Ee	SA	ZRC 2.5507b	24.2
	Enhydris punctata	Ep	SA	ZRC 2.3554	24.2
	Cerberus rynchops	Ce	SA	MNHN-RA-1998.8583	35.3
	Homalopsis buccata	Hb	SA	ZRC 2.6411	24.2
	Erpeton tentaculatum	Et	SA	GD pers. coll.	7.5
	Bitia hydroides	Bh	M	ZRC 2.4374	20.9
	Fordonia leucobalia	Fl	SA	MNHN-RA-1912.26	33.2
	Cantoria violacea	Cv	SA	ZRC 2.3672	20.8
Lamprophiidae	*Mimophis mahfalensis*	Mm	T	MRSN R3171	24.7
	Atractaspis irregularis	Ai	F	MNHN 1999.9129	7.6
Elapidae	*Micrurus lemniscatus*	Ml	T	MNHN 1997.2353	7.6
	Naja nivea	Nn	T	AH MS 68	28.5
	Hydrophis elegans	He	M	MNHN-RA-0.1879	30.7
	Enhydrina schistosa	Es	M	ZRC 2.2043	20.8
	Astrotia stokesii	As	M	ZRC 2.2032	20.8

	Hydrophis major	Hm	M	MNHN 1990 4557	44.8
	Hydrophis ornatus	Ho	M	MNHN-RA-1994.6997	36
	Pelamis platurus	Pp	M	AH MS 64	31.9
	Aipysurus duboisii	Ad	M	MNHN-RA-1990.4519	41
	Aipysurus eydouxii	Ae	M	MNHN-RA-0.7704	40.2
	Microcephalophis gracilis	Mg	M	ZRC 2.2155	20.8
Natricidae	*Thamnophis sirtalis*	Ta	T	GD pers. coll.	7.5
Colubridae	*Chrysopelea ornata*	Co	A	MCZ R-177291	14.9
	Hierophis gemonensis	Hg	T	AH Unnumb	23.4
	Hierophis viridiflavus	Hv	T	AH Unnumb	19.2
	Dispholidus typus	Dt	A	AH Unnumb	32
	Boiga dendrophila	Bd	A	AH MS 102	18.2
	Dasypeltis sp.	Ds	A	MCZ 71877	14.9
	Coronella austriaca	Ca	T	AH MS 51	21.6


Scolecophidia
Typhlophis squamosus (f)
Rhinotyphlops schlegelii (f)
Cylindrophis ruffus (f)
Uropeltis pulneyensis (f)
Anilius scytale (f)
Alethinophidia
Booidae
Python regius (t)
Boa constrictor (a)
Candoia sp. (t)
Corallus hortulanus (a)
Eunectes murinus (sa)
Macrostomata
Acrochordus granulatus (sa)
Pareas margaritophorus (a)
Crotalus atrox (t)
Agkistrodon contortrix (t)
Enhydris enhydris (sa)
Enhydris punctata (sa)
Cerberus rynchops (sa)
Homalopsis buccata (sa)
Erpeton tentaculum (sa)
Bitia hydroides (m)
Fordonia leucobalia (sa)
Cantoria violacea (sa)
Mimophis mahfalensis (t)
Atractaspis irregularis (f)
Micrurus lemniscatus (t)
Naja nivea (t)
Aipysurus duboisii (m)
Aipysurus eydouxii (m)
Hydrophiidae
Microcephalophis gracilis (m)
Hydrophis elegans (m)
Enhydrina schistosa (m)
Astrotia stokesii (m)
Pelamis platurus (m)
Hydrophis major (m)
Hydrophis ornatus (m)
Thamnophis sirtalis (t)
Colubridae
Chrysopelea ornata (a)
Hierophis gemonensis (t)
Hierophis viridiflavus (t)
Coronella austriaca (t)
Dispholidus typus (a)
Dasypeltis sp. (a)
Boiga dendrophila (a)

1057

Fig. 1. Schematic phylogenetic relationships of snakes sampled in the study (modified from Pyron et al., 2011; Hsiang et al., 2015; Lee and Scanlon, 2002). Principal ecology/habitat: fossorial (f), terrestrial (t), arboreal (a), semi-aquatic (sa), marine (m).

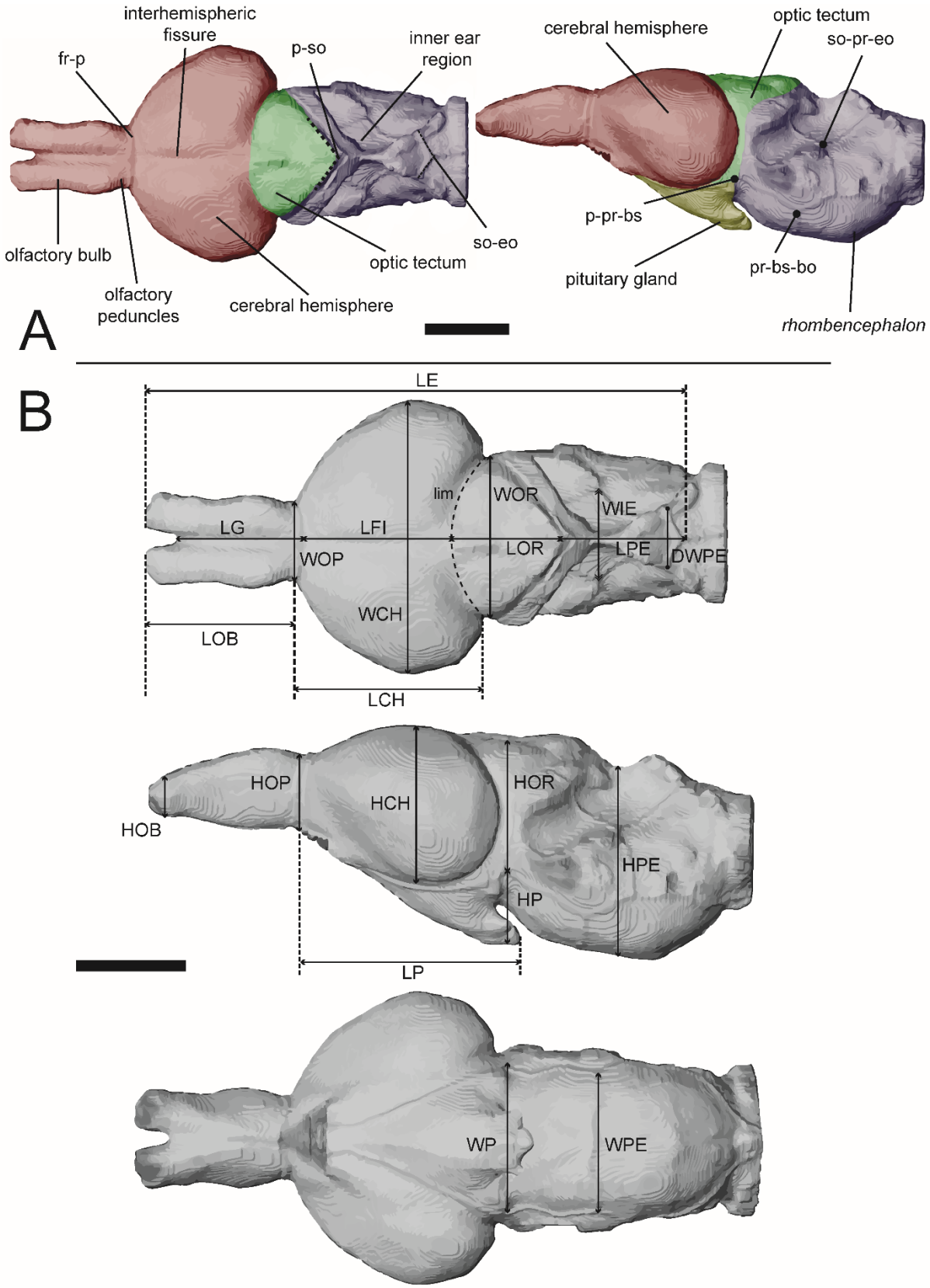

A
interhemispheric fissure
fr-p
p-so
inner ear region
olfactory bulb
olfactory peduncles
cerebral hemisphere
optic tectum
so-eo
cerebral hemisphere
optic tectum
so-pr-eo
p-pr-bs
pituitary gland
pr-bs-bo
rhombencephalon
B
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Fig. 2. Reconstructed brain endocast of *Enhydris punctata* (Homalopsidae) **(A)**, Illustration of the major structures seen in dorsal and left lateral views: telencephalon (red), diencephalon (yellow), mesencephalon (green), rhombencephalon (purple); **(B)**, Illustration of the various measurements defined in Material and Method and taken in dorsal, left lateral and ventral views. **Abbreviations: fr-p**, fronto-parietal suture; **lim**, groove between the optic tectum and the cerebral hemispheres; **p-pr-bs**, triple point formed by the sutures between the parietal, prootic and basisphenoid; **p-so**, parietal-supraoccipital suture; **pr-bs-bo**, triple point formed by the suture between the prootic, basisphenoid and basioccipital; **so-eo**, supraoccipital-exoccipital suture; **so-pr-eo**, triple point formed by the sutures of the supraoccipital, prootic and exoocipital; **DWPE**, Dorsal width of the posterior end of the brain endocast; **HCH**, Maximal height of the cerebral hemisphere; **HOB**, Height of the main olfactory bulb; **HOP**, Height of the olfactory peduncle; **HOR**, Height of the optic tectum; **HP**, Height of the pituitary bulb; **HPE**, Height of the posterior part of the brain endocast; **LCH**, Lateral expansion of the cerebral hemispheres; **LE**, Length of the brain endocast; **LFI**, Length of the interhemispheric fissure; **LG**, Length of the groove between olfactory bulbs; **LOB**, Length of the olfactory bulbs; **LOR**, Length of the optic tectum; **LP**, Length of the pituitary bulb; **LPE**, Length of the posterior part of the brain endocast; **WCH**, Maximal width of the cerebral hemispheres; **WIE**, Width in the inner ear region; **WOP**, Width of the olfactory peduncles; **WOR**, Maximal width of the optic tectum; **WP**, Width in the pituitary gland region; **WPE**, Width of the ventral part of the brain endocast. Scale bar equals to 2 mm.

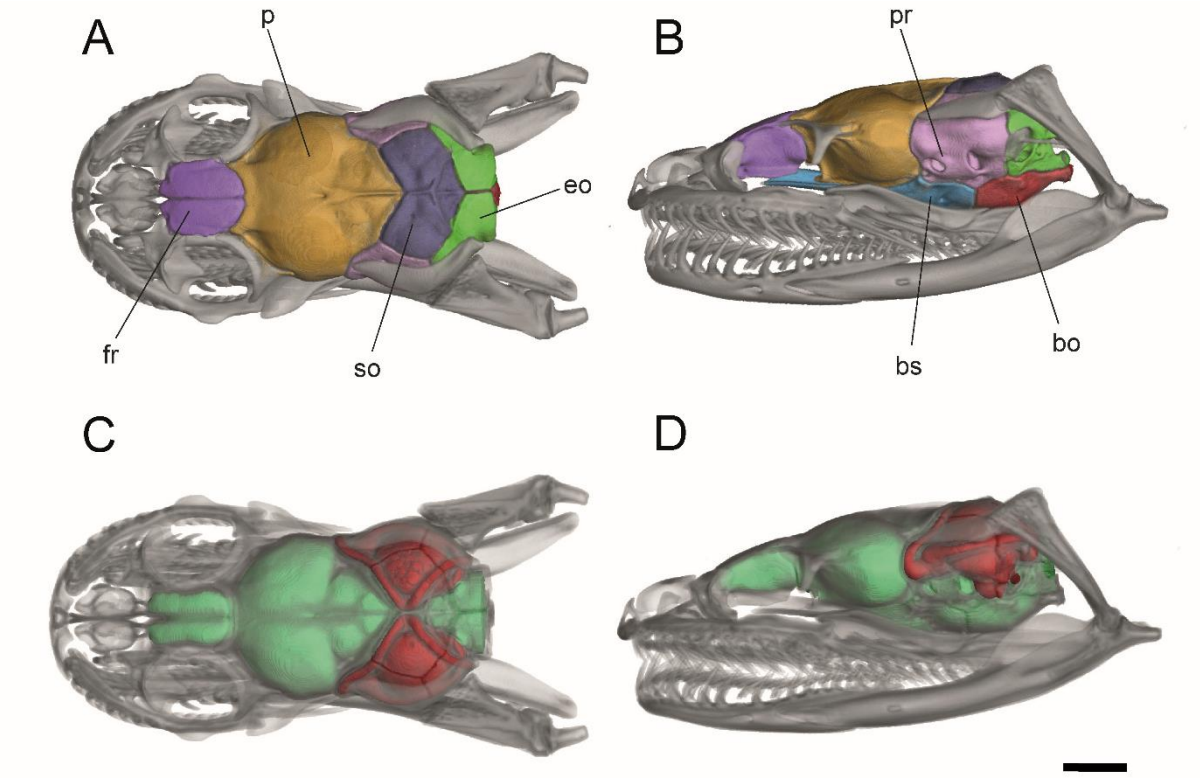

Fig. 3. Skull of *Enhydris punctata* (Homalopsidae) in dorsal (A) and left lateral (B) views showing the bones surrounding the brain endocast; (C-D) with bones rendered transparent to reveal the brain endocast (green) and the inner ear (red). **Abbreviations: bo**, basioccipital; **bs**, basisphenoid; **eo**, exoccipitals; **fr**, frontal; **p**, parietal; **pr**, prootics; **so**, supraoccipitals. Scale bar equals 2 mm.

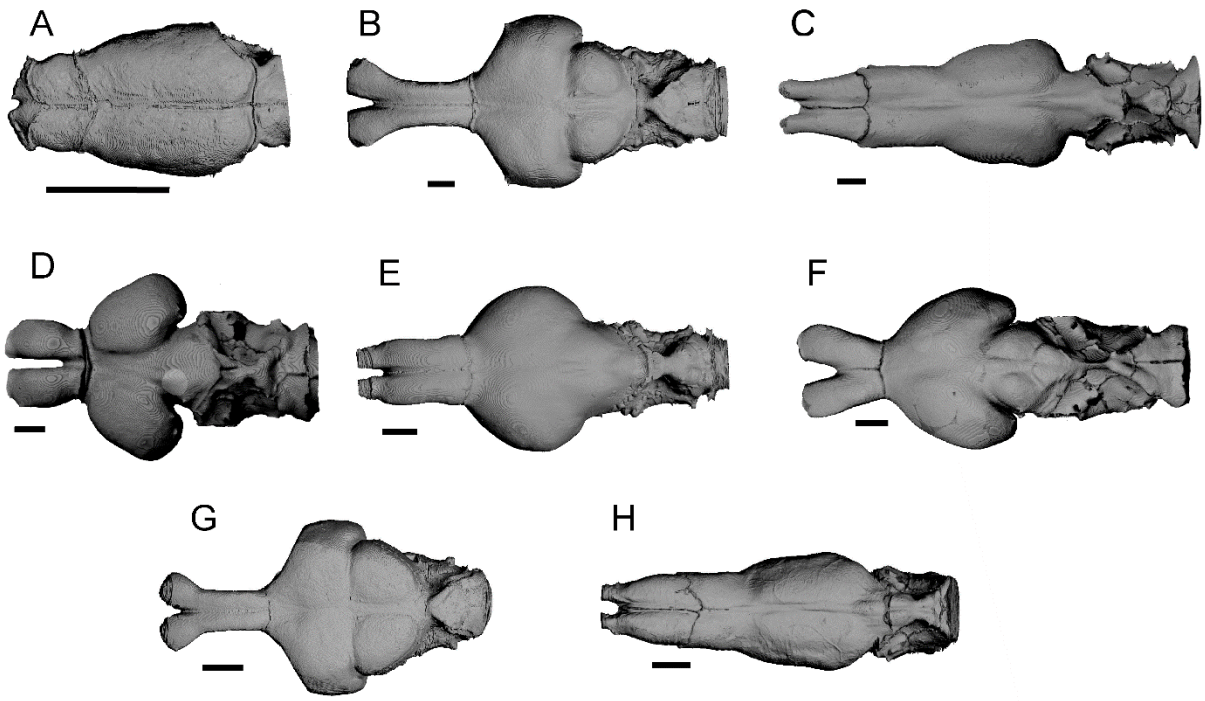

Fig. 4. Brain endocasts in dorsal view of **(A)** *Typhlophys squamosus* (Typhlopidae); **(B)** *Hierophis viridiflavus* (Colubridae); **(C)** *Cylindrophis ruffus* (Cylindrophiidae); **(D)** *Acrochordus granulatus* (Acrochordidae); **(E)** *Eunectes murinus* (Boidae); **(F)** *Homalopsis buccata* (Homalopsidae); **(G)** *Chrysopelea ornata* (Colubridae); **(H)** *Anilius scytale* (Aniliidae). Scale bars equal 1mm.

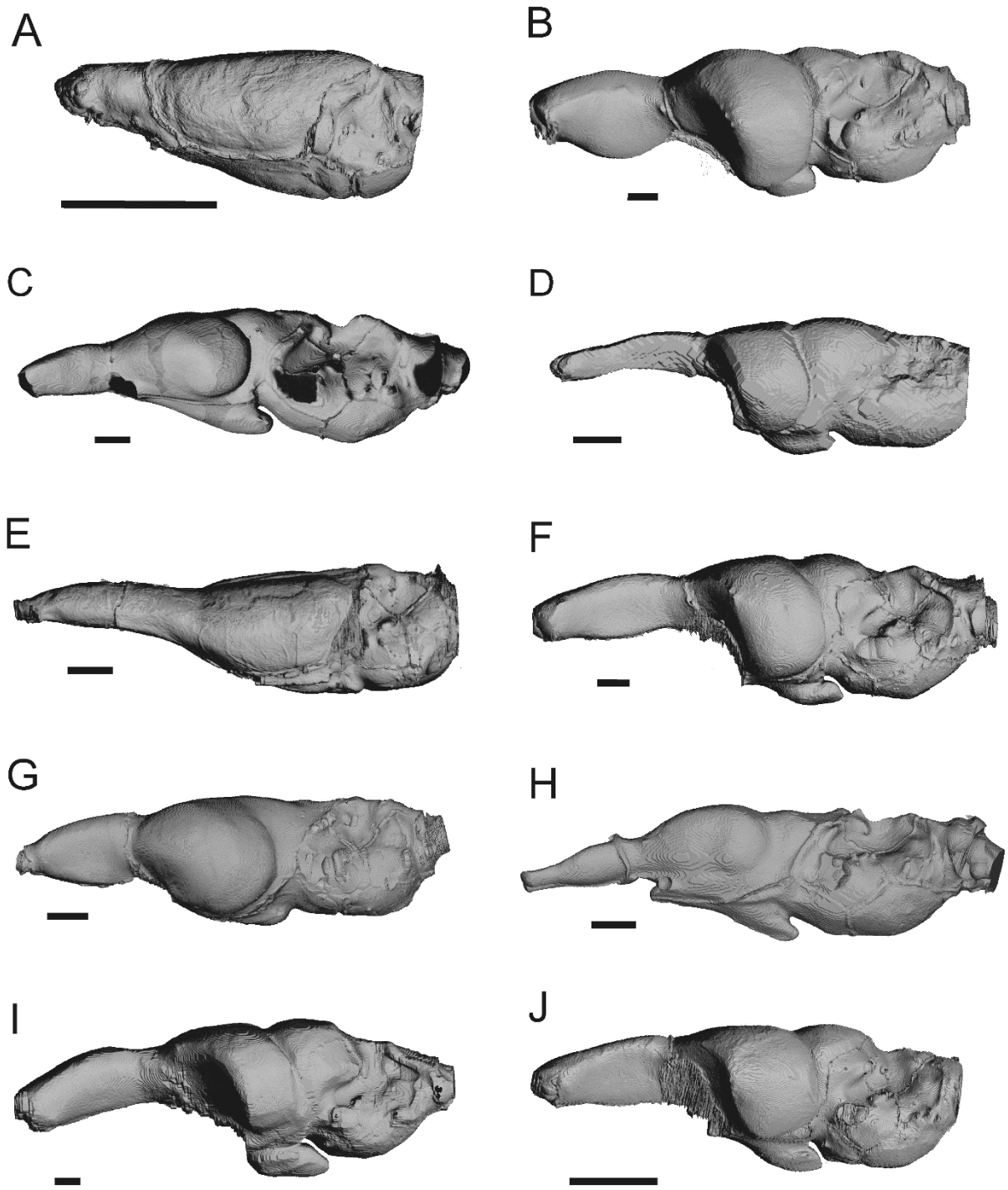

Fig. 5. Brain endocasts in left lateral view of **(A)** *Typhlophys squamosus* (Typhlopidae); **(B)** *Boiga dendrophila* (Colubridae); **(C)** *Homalopsis buccata* (Homalopsidae); **(D)** *Mimophis mahfalensis* (Lamprophiidae); **(E)** *Anilius scytale* (Aniliidae); **(F)** *Hierophis viridiflavus* (Colubridae); **(G)** *Eunectes murinus* (Boidae); **(H)** *Enhydrina schistosa* (Elapidae); **(I)** *Dispholidus typus* (Colubridae); **(J)** *Thamnophis sirtalis* (Natricidae). Scale bars equal 1mm.

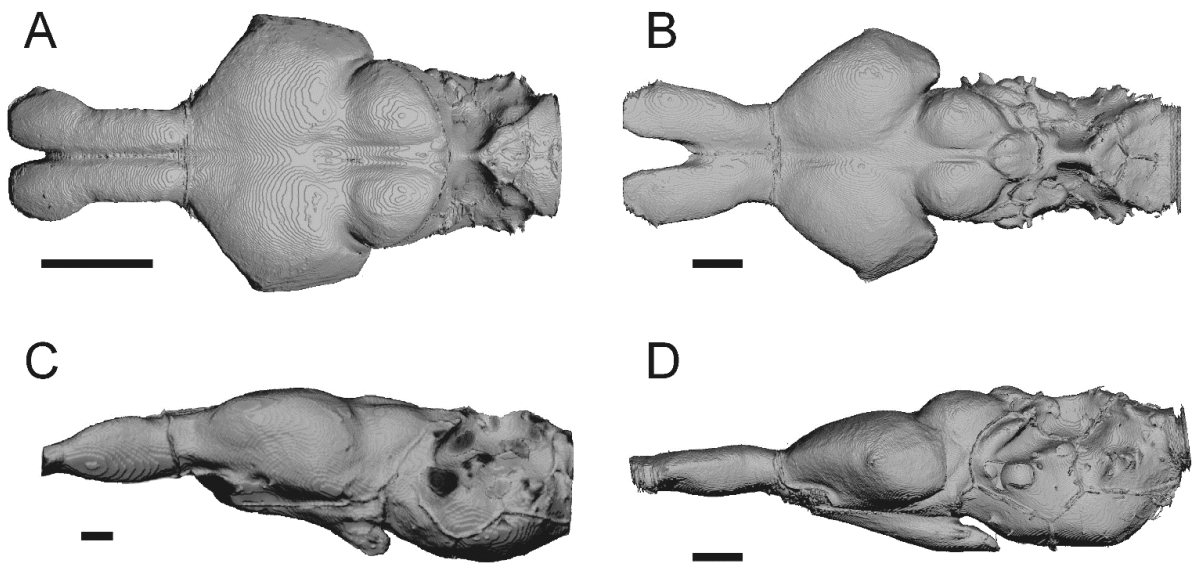

Fig. 6. Brain endocasts in dorsal (upper row) and left lateral (lower row) views of **(A)** *Thamnophis sirtalis* (Natricidae); **(B)** *Erpeton tentaculatum* (Homalopsidae); **(C)** *Hydrophis major* (Elapidae); **(D)** *Erpeton tentaculatum*. Scale bars equal 1mm.

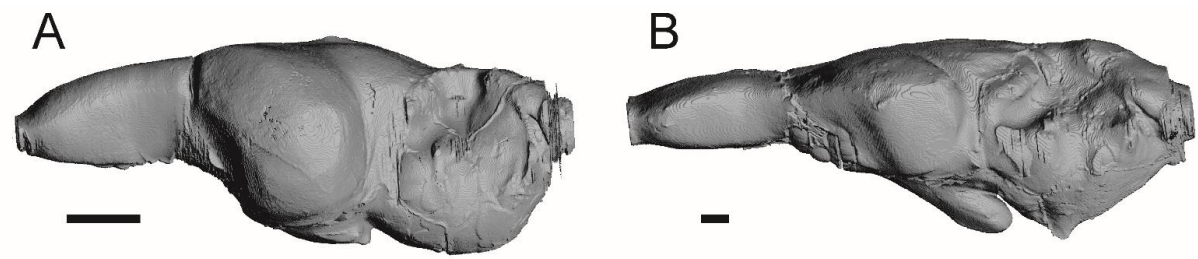

Fig. 7. Brain endocasts in left lateral view of **(A)** *Boa constrictor* (Boidae); **(B)** *Crotalus atrox* (Viperidae). Scale bars equal 1mm.

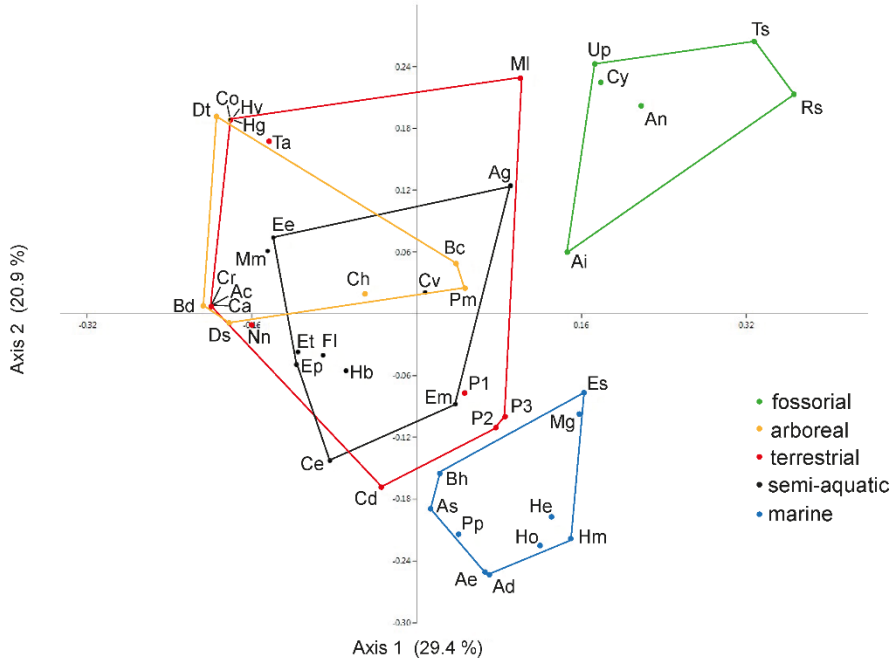

Fig. 8. Results of the principal coordinate analysis performed on the snake brain endocast characters (Supplementary Data S3). See Table 1 for name abbreviations.

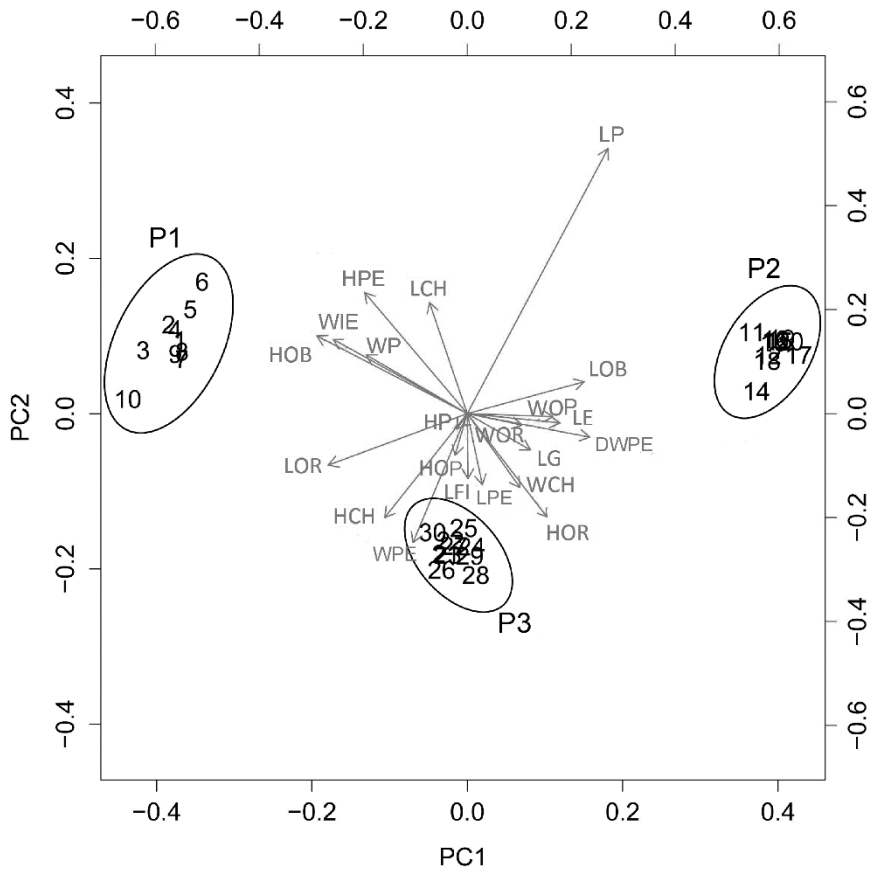

Fig. 9. Results of the principal component analysis performed on the brain endocast variables for three *Python regius* specimens, (P1) smaller specimen, (P3) intermediate specimen, (P2) largest specimen. Scatter plot illustrating the position of the different specimens on the first two principal components. **Abbreviations: DWPE**, Dorsal width of the posterior end of the brain endocast; **HCH**, Maximal height of the cerebral hemisphere; **HOB**, Height of the main olfactory bulb; **HOP**, Height of the olfactory peduncle; **HOR**, Height of the optic tectum; **HP**, Height of the pituitary bulb; **HPE**, Height of the posterior part of the brain endocast; **LCH**, Lateral expansion of the cerebral hemispheres; **LE**, Length of the brain endocast; **LFI**, Length of the interhemispheric fissure; **LG**, Length of the groove between olfactory bulbs; **LOB**, Length of the olfactory bulbs; **LOR**, Length of the optic tectum; **LP**, Length of the pituitary gland; **LPE**, Length of the posterior part of the brain endocast; **WCH**, Maximal width of the cerebral hemispheres; **WIE**, Width in the inner ear region; **WOP**, Width of the olfactory

peduncles; **WOR**, Maximal width of the optic tectum; **WP**, Width in the pituitary gland region; **WPE**, Width of the ventral part of the brain endocast.

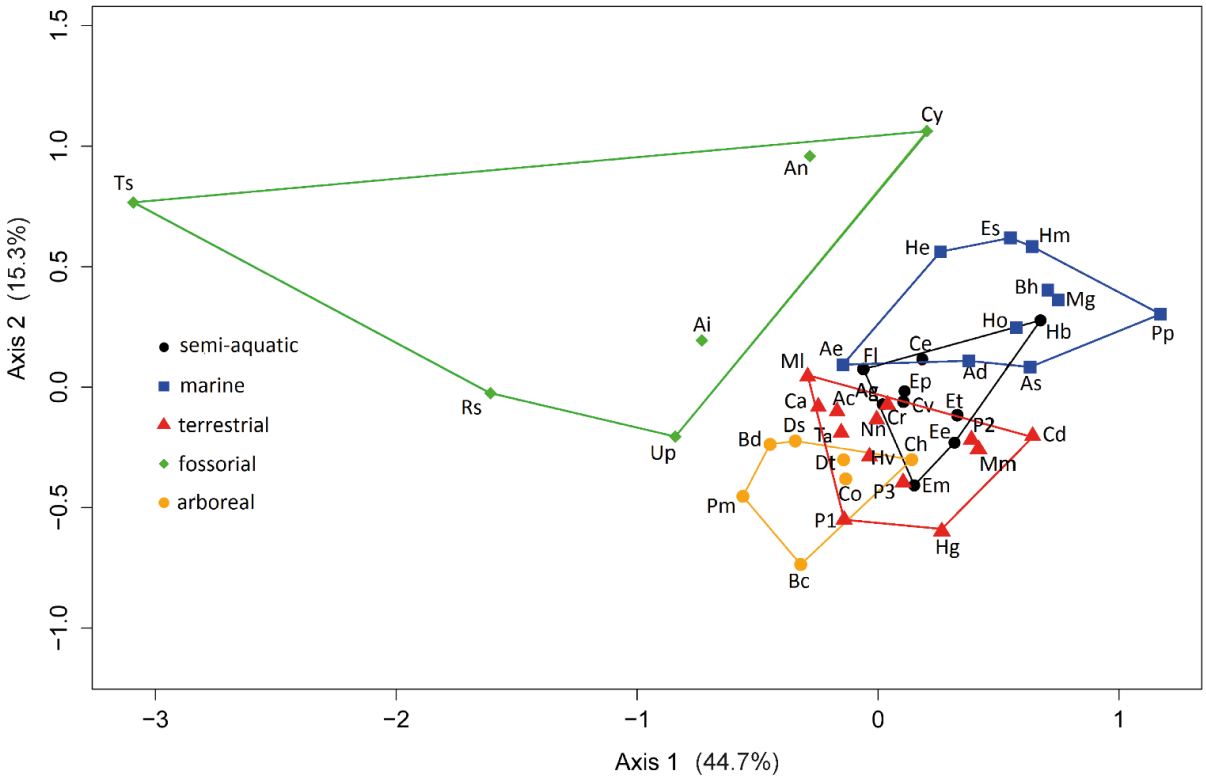

Fig. 10. Results of the principal component analyses performed on the snake brain endocast variables of the 45 specimens. Scatter plot illustrating the position of the different species on the first and second principal components and figuring the different ecologies. See Table 1 for name abbreviations.

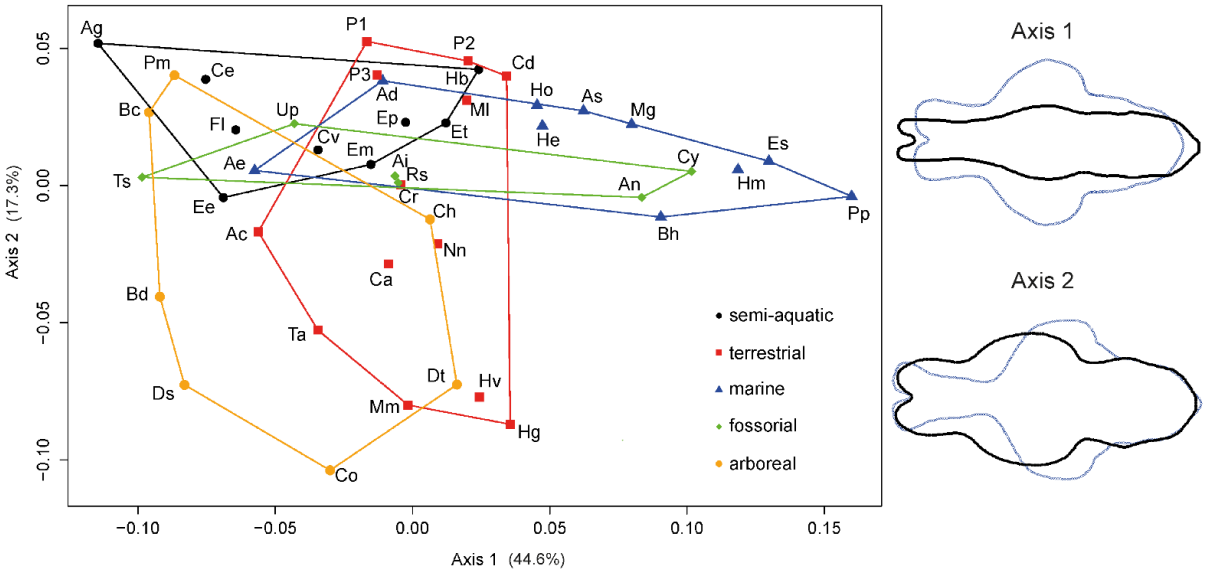

Fig. 11. Results of the principal component analyses performed on the snake brain endocast outline curves in ventral view. The blue and dark dotted lines indicate respectively the low and high values along the two axes. See Table 1 for name abbreviations.

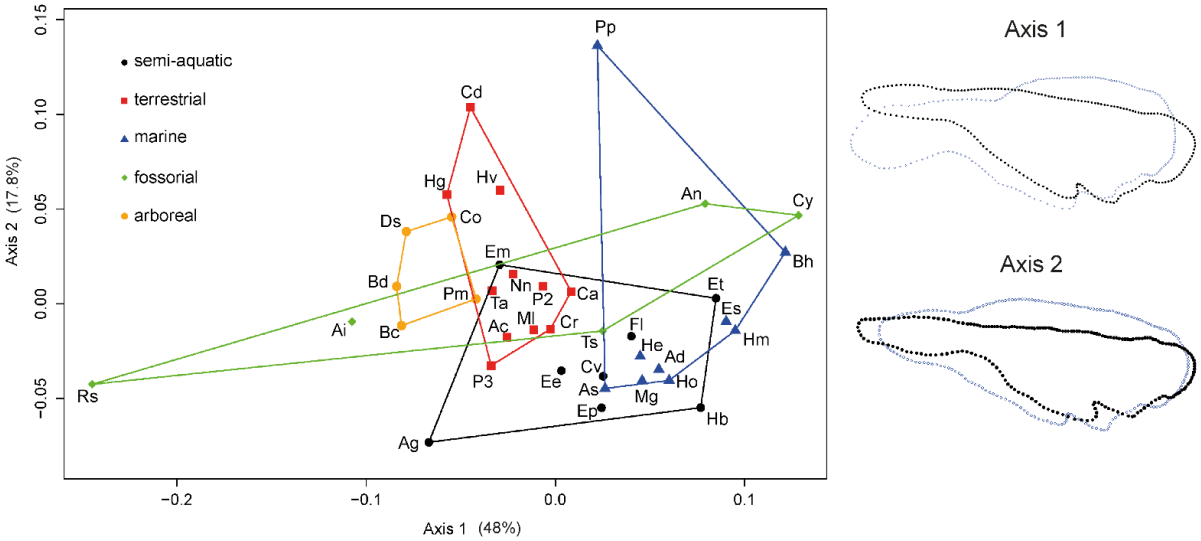

Fig. 12. Results of the principal component analyses performed on the snake brain endocast outline curves in lateral view. The blue and dark dotted lines indicate respectively the low and high values along the two axes. See Table 1 for name abbreviations.