Inside the head of snakes: influence of size, phylogeny, and sensory ecology on endocranium morphology

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1 **Inside the head of snakes: influence of size, phylogeny, and sensory ecology on endocranium**

2 **morphology.**

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14 **Abstract**

15 Environmental properties, and the behavioral habits of species impact sensory cues available for

16 foraging, predator avoidance and inter/intraspecific communication. Consequently, relationships

17 have been discovered between the sensory ecology and brain morphology in many groups of

18 vertebrates. However, these types of studies have remained scare on snake. Here, we investigate

19 the link between endocranial shape and the sensory-related ecology of snakes by comparing 36

20 species of snakes for which we gathered six sensory-ecology characteristics. We use µCT scanning

21 and 3D geometric morphometrics to compare their endocranium in a phylogenetically informed

22 context. Our results demonstrate that size is a major driver of endocranial shape, with smaller

23 species tending to maximize endocranial volume using a more bulbous shape, while larger species

24 share an elongate endocranial morphology. Phylogeny plays a secondary role with more derived

25 snakes diverging the most in endocranial shape, compared to other species. The activity period

26 influences the shape of the olfactory and optic tract, while the foraging habitat impacts the shape

27 of the cerebellum and cranial nerve regions: structures involved in orientation, equilibrium, and

28 sensory information. However, we found that endocranial morphology alone is not sufficient to

29 predict the activity period of a species without prior knowledge of its phylogenetic relationship.

30　Our results thus demonstrate the value of utilizing endocranial shape as complementary

31　information to size and volume in neurobiological studies.

32　**Key words:** 3D geometric morphometrics, ecological morphology, allometry, snake endocranium,

33　activity period, foraging habitat

34　**Declarations**

48

Introduction

Endocranial shape has been extensively used as a proxy for the brain shape, to compare or infer the ecology of both extant and extinct species (for a review, see Balanoff & Bever, 2017); including habitat (Allemand et al. 2017), sensory abilities (Lautenschlager et al. 2012; Holloway et al. 2013; Carril et al. 2016) and behavior (Balanoff et al. 2016; Bertrand et al. 2019; Macrì et al. 2019). However, endocranial morphology could also be used to make more precise inferences regarding the sensory ecology of elusive (Iwaniuk et al. 2020) or fossil species, if we were able to recognize a direct link between sensory-relevant aspects of the ecology of extant species and the shape of their endocranium. An extensive comparative study of the brain shape of fish has demonstrated a relationship between brain shape and precise details of the ecology such as microhabitat use and diet (Kotrschal et al. 1998) supporting the hypothesis that brain shape is adaptive. Snakes, like fish, have a compartmentalized brain (Naumann et al. 2015), with clearly identifiable sensory-associated structures such as the olfactory bulbs and optic tectum, and because the brain sits tightly within the endocranial cavity, the latter is a good proxy for brain shape (Starck 1979; Nieuwenhuys et al. 1998, pers. obs.). The main goal of this study is to determine if there are potential ecological drivers of endocranial shape in snakes, by statistically testing for relationships between sensory ecology and endocranial morphology. We focus on snake' species that use aquatic habitats to varying extents as water requires specific sensory adaptations, especially for secondarily aquatic animals. We worked on a phylogenetically large sample of species to ensure our results reflect adaptive changes due to shared ecological constraints.

Snakes have an arsenal of sensory modalities that they can use. They are known to use vomerolfaction using their forked tongue to sample the environment (Daghfous et al. 2012), but they can also process olfactory and gustative cues, even though they rely more on the first modality. Despite their lack of visual acuity and despite popular belief, snakes heavily rely on visual cues to sense their environment (Czaplicki and Porter 1974; Drummond 1985; Hart et al. 2012), but they also use thermal cues (Newman and Hartline 1982; De Cock Buning 1983; Krochmal et al. 2004; Ebert and Westhoff 2006; Ebert et al. 2007), chemical cues (Shine et al. 2004a; Young et al. 2008; Smargiassi et al. 2012), airborne and/or waterborne acoustic cues (Randall and Matocq 1997; Young 2003, 2007; Friedel et al. 2008), water motion through mechanoreception (Povel and Van Der Kooij 1997; Westhoff et al. 2005; Catania et al. 2010;

79   Crowe-Riddell et al. 2016) and even phototaxis (Crowe-Riddell et al. 2019) (albeit the latter may

80   not be mediated in the brain). There is some evidence that snakes can also adapt their sensory

81   modalities depending on properties of their environment, behavior or diet, if those impact the

82   availability or reliability of the sensory cues (Burghardt and Ford 1993; Vincent et al. 2005; Cooper

83   2008; Schwenk 2008; Hart et al. 2012; Crowe-Riddell et al. 2016; Kutsuma et al. 2018). Our main

84   hypothesis is that the morphology of the endocranium, and its sensory structures is related to their

85   ecology of species as the latter seems to impact their sensory modalities. Studies quantifying the

86   sensory modalities used by snakes are scarce (Burghardt and Ford 1993) so we cannot directly test

87   how the preferred senses of species are related to the shape of the associated sensory area.

88   Therefore, we defined six ecological and behavioral factors that could impact the sensory

89   modalities of snakes and consequently lead to change in their endocranium shape: diet type,

90   foraging strategy, foraging habitat, main habitat, activity period, and dimensionality of the trophic

91   interaction (Table 1).

92   Dietary preferences in fish, and especially the importance of piscivory, correlate with the size

93   and shape of the brain, and more specifically the sensory-related parts of the brain such as the

94   olfactory bulbs and optic lobes (Kotrschal et al. 1998). In birds, diet and brain regions are also

95   related, but whereas in fish this seems to be based on perception/detection of a prey, in birds it is

96   more related to the complexity of the food manipulation (Gutiérrez-Ibáñez et al. 2010). Snakes not

97   only have a very diverse dietary range, but they also use their head to manipulate and swallow

98   their prey (Moon et al. 2019). Some crustacean-eating snakes such as *Fordonia leucobalia*,

99   *Cantoria violacea* and *Gerarda prevostiana* even show complex manipulation behavior (Jayne et

100   al. 2018). We distinguished 5 diet types depending on the media in which prey live and the

101   potential manipulation cost: generalists are species of snake that eat both aquatic and non-aquatic

102   prey, and four specialists are species that eat exclusively certain types of aquatic or semi-aquatic

103   prey (i.e., fish, crustacean, fish and crustaceans, fish and amphibians).

104   The foraging strategy is also known to impact the preferred sensory cues used by snakes to

105   locate their prey; ambush predators tends to rely more upon vision (Czaplicki and Porter 1974) or

106   mechanoreception (Westhoff et al. 2005; Catania et al. 2010) whereas active foragers track their

107   prey using chemical cues (Cooper 2008; Smargiassi et al. 2012). We divided foraging strategy in

108   3 categories: species actively chasing prey, ambushing, or doing both alternatively.

109   The properties of the environment in which an animal behaves influence the availability and
110 predictability of the sensory cues (Thewissen and Nummela 2008; Stevens 2013). For instance,
111 the chemical diffusion under water is slower but more traceable than in air. The light level and
112 spectrum decrease with depth under water, while sound is more efficiently propagated in water
113 than in air. Different media also retain cues of different nature; mud and slime retain more
114 hydrophobic odorant; water carries hydrophilic molecules and air small volatile compounds. We
115 divided the environment of species in two categories: foraging habitat and main habitat. We
116 defined the main habitat depending on the substrate in which the species spends most of its time
117 and divided it in 3 categories: aquatic species are the ones rarely found outside of water, semi-
118 aquatic species are found both on land and under water, and mud/fossorial species. The foraging
119 habitat is the medium in which species forage most of the time (i.e., land, water, or both). Some
120 species can forage in one media but rest in another, which would require more adaptability of the
121 sensory modalities of these species, whereas species that only are foraging in one media should
122 demonstrate more specialization.

123   The activity pattern (i.e., nocturnal, diurnal, cathemeral) is another aspect of the ecology of
124 species that impacts their sensory preferences. Nocturnality and low light environments (scotopic)
125 strongly impact the brain shape of vertebrates (Barton et al. 1995; Kaas 2017) in two alternative
126 strategies. Some species of birds or fish demonstrate a reduction of the visual system that is often
127 associated with reduction in size of the visual apparatus and the increase of another sensory
128 pathway (e.g. olfaction or mechanoreception); or alternatively show an increase of parts or the
129 whole visual apparatus to allow more light reception (Kotrschal et al. 1998; Kaas 2017). Diurnal
130 snakes are expected to rely more heavily on visual cues than cathemeral species that might use a
131 combination of sensory information.

132   Dimensionality and complexity of the foraging habitat have been correlated with shape and
133 size variations of some brain regions (i.e. cerebellum) in fish, birds and some squamates (Kotrschal
134 et al. 1998; Kaas 2017; Yopak et al. 2017; Macrì et al. 2019). Instead of considering habitat
135 dimensionality, we considered the dimensionality of the trophic interaction (Pawar et al. 2012),
136 which is the number of dimensions the predator is using to detect prey (e.g. 2D for grazers and 3D
137 for flying insect catchers). In a foraging context, the predator must quickly gather and process
138 reliable sensory information and adjust his behavior consequently, which might require specific

139 adaptation for snakes foraging in 3D environments. We considered snakes foraging in open water
140 to have a 3D interaction with their prey, and substrate-surface foraging species to have 2D trophic
141 interactions (e.g. crevice-foraging, coral reef probing or bottom-dwelling fish specialists).

142   These six ecological and behavioral characteristics have been demonstrated to be related to
143 the endocranial and/or brain shape in other groups of vertebrates. Previous work have highlighted
144 a link between brain or endocasts and evolutionary history in birds, fish and some squamates
145 (Kotrschal et al. 1998; Allemand et al. 2017; Yopak et al. 2017). However, this link becomes
146 weaker when the phylogenetic distance becomes small and when species are closely related, but
147 ecological influences become stronger (Kotrschal et al. 1998). To our knowledge, no work
148 attempted to gather all this information to create a complete ecological identity for a large number
149 of species and relate it to the endocranial shape. Because we do not only focus on the whole shape
150 of the endocranium, but also on its different regions, we summarized (see Table 1) our predictive
151 factors (with definitions), the way they could impact sensory cues, and the endocranial area that
152 might be impacted. For this study, we compared the endocranium of 36 snake species that are
153 geographically, phylogenetically, and ecologically diverse and show different degrees of aquatic
154 habits. We used μ-CT (computed tomography) scans of museum specimens; we characterize the
155 endocranium shape using a 3D geometric morphometric approach. This method allows us to
156 transform volume into a size and shape component and offers a more comprehensive approach to
157 investigate the morphology of the endocranium (Kawabe et al. 2013; Marugán-Lobón et al. 2016).
158 We used phylogenetic comparative methods to test for the relationship between sensory ecology
159 and shape, and a reclassification algorithm to assess whether the endocranium shape could be used
160 to infer the sensory ecology of elusive or fossil snake species.

161 ***Table 1:*** *Ecological characteristics of interest and hypothetical impacts on the sensory modalities and endocranial shape of snakes.*

| Ecological Factor	Categories	Comment	Impact on sensory modalities	Potentially impacted endocranial area
Diet type	Fish specialist	Some species of snakes are specialized in specific fish species (e.g., *L. colubrina*)	Fish specialists need to accurately detect and recognize their specific prey either through olfactory or visual cues (Kutsuma et al. 2018).	
	Crustacean specialist	Can be either fully aquatic or semi-aquatic. Some species specialized in freshly molted or hard crustaceans	Crustacean specialists rely on chemical cues to detect their prey (Mark Waters and Burghardt 2005) and show complex manipulation (Jayne et al. 2002; Noonloy et al. 2018) which may be reflected in their olfactory tract and cerebellum.	➤ **optic tract** ➤ **olfactory tract**
	Fish and crustaceans	Mostly semi-aquatic species	Fish and amphibians are fast moving preys that are generally detected by snakes using vision and/or mechanoreception (Czaplicki and Porter 1974; Camilleri and Shine 1990; Catania et al. 2010) while fish and crustacean eaters can rely on chemo- and mechanoreception, but also on vision.	➤ **cerebral hemispheres** ➤ **cerebellum** ➤ **cranial nerves**
	Fish and amphibians	Mostly semi-aquatic species		
	Generalist **G**	More opportunistic species. Can catch either aquatic or non-aquatic prey. Usually more terrestrial species	Generalist snakes are expected to show no specialization related to diet as they must detect and catch preys in two different media and are opportunistic.	
Foraging strategy	Active **A**	Tracking a prey	Rely on persistent cues such as chemical and/or visual cues.	➤ **optic tract** ➤ **olfactory tract**
	Sit-and-wait **SW**	Fast triggering cue in a close range	Rely more on mechanoreception or visual cues.	➤ **cerebral hemispheres**
	Opportunistic **O**	Using one or the other technique	Same as generalists.	➤ **cranial nerves (trigeminal)**
Foraging habitat	Water	- Odorant: hydrophilic molecules, low diffusion speed, high predictability of the source position - Visual cues: light level and spectrum decrease with depth and turbidity, high refractive index - Acoustic cues and mechanoreception: high velocity and impedance	Fully aquatic species rely more on chemical cues if active forager and acoustic/mechanoreception if sit-and-wait (Camilleri and Shine 1990). They use visual cues to locate preys (Kutsuma et al. 2018) and strike at moving objects (Czaplicki and Porter 1974; Catania et al. 2010). Depending on associated dimensionality, aquatic snakes might need to process sensory cues in 3D.	➤ **olfactory tract** ➤ **cerebral hemispheres** ➤ **cerebellum**
	Land	- Odorant: volatile molecules, high diffusion speed, impaired predictability - Visual cues: good during daylight if no obstruction, low refractive index - Acoustic cues and mechanoreception: low velocity and impedance	Mostly terrestrial snakes probably rely on vision and vibrational or airborne acoustic cues during foraging as these are more accurate and traceable on land (Young et al. 2008).	➤ **optic tract** ➤ **cranial nerves (trigeminal & VIII)**

	Both �(grey square)	Difference of refractive index when changing media	Requires adaptability in the sensory modalities because of the difference in the signal nature and propagation in different media.	
Main habitat	Aquatic ▪(cyan square)	See "water" in Foraging habitat	Some aquatic species use vision to find mate (Shine 2005). Sea snakes show a diversification of the visual pigments suggesting that vision is important in their ecology (Simões et al. 2020). Depending on associated dimensionality, might need to process sensory cues in 3D.	➢ **olfactory tract** ➢ **cerebral hemispheres**
	Semi-aquatic ◳(half-filled square)	Change of refractive index when changing media	Some semi-aquatic snakes evolved accommodation mechanisms suggesting that vision is important on both land and in water (Schaeffel and de Queiroz 1990; Schaeffel and Mathis 1991).	➢ **cerebellum** ➢ **optic tract**
	Mud ▪(brown square)	Negative impact on vision, long persistence time of chemical cues, acoustic or mechanical cues probably less reliable	Mud snakes must rely on vomerolfaction or olfaction, and their optic tract might have regressed compared to the aquatic and semi-aquatic species.	➢ **cranial nerves (trigeminal & VIII) and otic area** ➢
Activity period	Diurnal ☀	Foraging during day light	Diurnal snakes have larger eyes (Liu et al. 2012; Da Silva et al. 2017) suggesting that they might heavily rely on vision.	➢ **optic tract**
	Nocturnal ☾	Foraging in low light environment	Two alternative strategies: - enlargement of visual system to capture more light - reduction of the visual system and reliance on chemical and/or mechanical cues.	➢ **olfactory tract** ➢ **cerebral hemispheres**
	Cathemeral ☀	Sporadic activity during the day or night	Requires the use of various cues depending on the light level.	
Prey-predator interaction dimensionality	**3D**	Foraging in open water	Requires precise stereo sensory information and a more complex signal processing which may be reflected in their cerebellum.	➢ **cerebral hemispheres**
	2D	Foraging along a substrate/surface	Simpler sensory cues to process	➢ **cerebellum** (Macrì et al. 2019)

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Material and Methods

Endocranium acquisition

We compared 36 species that cover both the phylogenetic and ecological diversity of snakes, from fully aquatic, highly specialized to generalist species (Fig. 1). We included 2-6 adult specimens per species (see list in Supplementary Material 1) from several museum collections (AMNH, FMNH, CAS). In total, the skulls of 98 specimens were scanned using the X-ray µCT-scanner (2010 GE phoenix v| tome|x s240 high-resolution microfocus computed tomography system, General Electric, Fairfield, CT, USA) at the Microscopy and Imaging Facility at the AMNH (New York, NY, USA). Scans were performed with a voltage between 100-150kV and current between 130-160µA for a voxel size between 15.6-57.4µm. The 3D reconstruction was performed using the software Phoenix datos|x2 and the subsequent segmentation was done using VGStudioMax v. 3.0 (Volume Graphics GmbH, Heidelberg, Germany). Previous studies used virtual/digital brain endocasts which are obtained by manually filling the endocranial space (Olori 2010; Balanoff et al. 2016; Carril et al. 2016; Allemand et al. 2017). This technique is widely used but raises many questions regarding the repeatability and biological accuracy of the obtained 3D objects, especially concerning the foramina and fenestrae (Balanoff et al. 2015). To avoid any bias or extrapolation, our landmarks were placed directly on the internal surface of the reconstructed skull (i.e. the endocranium) using the software MorphoDig 1.2 (Lebrun 2017). Some areas related to sensory structures of the brain are easily identifiable in the endocranium and were used to test our hypotheses, namely the olfactory tract, the cerebral hemispheres, the optic tract, the cerebellum, and the area where the cranial nerves meet the brain, that we named cranial nerve area (Supplementary Material 2b.). This area is also shaped by the presence of the inner ear and can give us a proxy for the shape of the otic capsule. We included the infundibulum and pituitary gland in our analyses despite these structures are not involve in the sensory system but rather in hormone secretions (Fig. 2).
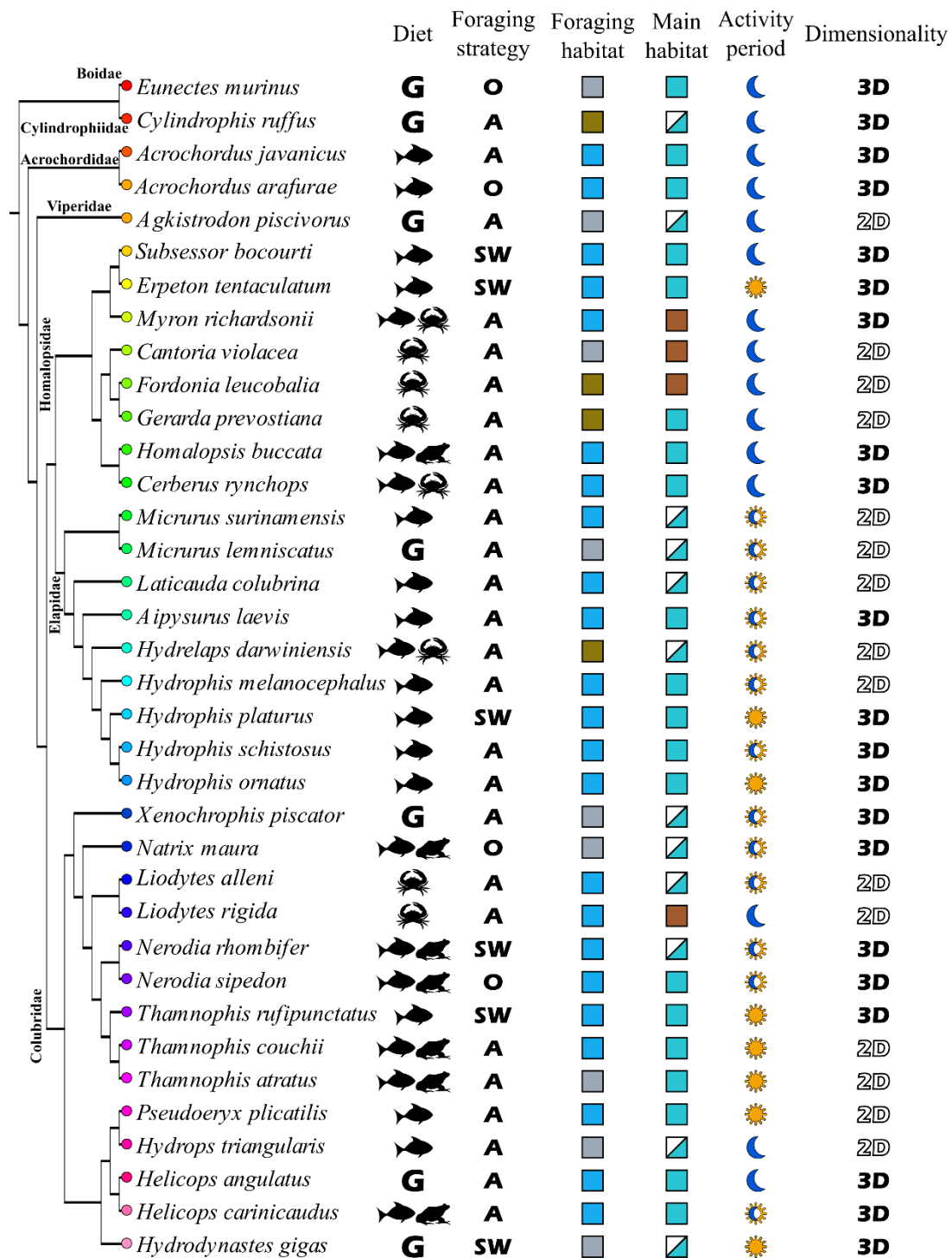
Fig. 1: Phylogeny of the 36 included snake species (reduced phylogeny from Pyron & Burbrink, 2014) *along with ecological and behavioral characteristics: diet type: generalist* **G**, *piscivorus* 🐟, *fish and amphibians* 🐟🐸, *crustaceans* 🦀, *fish and crustaceans* 🐟🦀; *foraging strategy: active* **A**, *sit-and-wait* **SW**, *opportunistic* **O**; *foraging habitat: land* ▪, *water* ▪, *both* ▪; *main habitat: aquatic* ▪, *mud* ▪, *semi-aquatic* ◪; *activity period: diurnal* ☀, *nocturnal* 🌙, *cathemeral* ☀; *dimensionality:* 2D *or* 3D

10

195   *Geometric morphometrics*

196   We created a template of the endocranium using a total of 848 landmarks: 73 anatomical

197   landmarks, 425 curve semi-landmarks and 350 surface semi-landmarks (Fig. 2, see Supplementary

198   Material 2). We placed the anatomical landmarks and curve semi-landmarks by hand on each

199   specimen. Then, we used the 'Morpho' package (Schlager 2015) to project and relax the surface

200   semi-landmarks of the template on each specimen. Finally, the curve and surface semi-landmarks

201   were allowed to slide on each specimen while minimizing the bending energy between the

202   specimen and the mean shape landmark configuration (Gunz and Mitteroecker 2013). To obtain a

203   mean shape for each species, we performed a Procrustes superimposition (GPA) of the specimens

204   of each species separately and we symmetrized the configurations using the function *procSym* of

205   the 'Morpho' package. We resized the obtained mean shape per species using their mean centroid

206   size and obtained our array of species configuration. Finally, we performed another Procrustes

207   superimposition on the species mean shapes using the function *gpagen* of the 'geomorph' package

208   (Adams et al. 2020). We used Procrustes coordinates as the shape variable to test our hypotheses.

209   Additionally, we performed a Principal Component Analysis (PCA) using the *plotTangentSpace*

210   function in 'geomorph' to test our hypothesis on a subset of variables, as inferential tests are

211   sensitive to the number of variables. We chose to test two subsets: 90% of the overall shape

212   variability (PC90: first 9 Principal Components) and the meaningful PC using the function

213   *getMeaningfulPCs* in the 'Morpho' package (mPC: 2 first PCs; Supplementary Material 3). For

214   the endocranial areas analyses, we used subsets of the mean species array and performed a GPA

215   using *gpagen* as we did for the whole endocranium (Fig. 2, Supplementary Material 2b.). We

216   performed the same statistical analysis on each area as we did for the whole endocranium.

217   *Sensory-related traits*

218   We defined each species characteristics based on the literature and on personal observations for

219   some less documented species (Fig. 1, see Supplementary Material 1 for a complete reference

220   source of these data). We summarized in Table 1 the list of the ecological and behavioral

221   characteristics we considered, along with the associated impact on the sensory modalities and the

222   area of the endocranium that could hypothetically be impacted.

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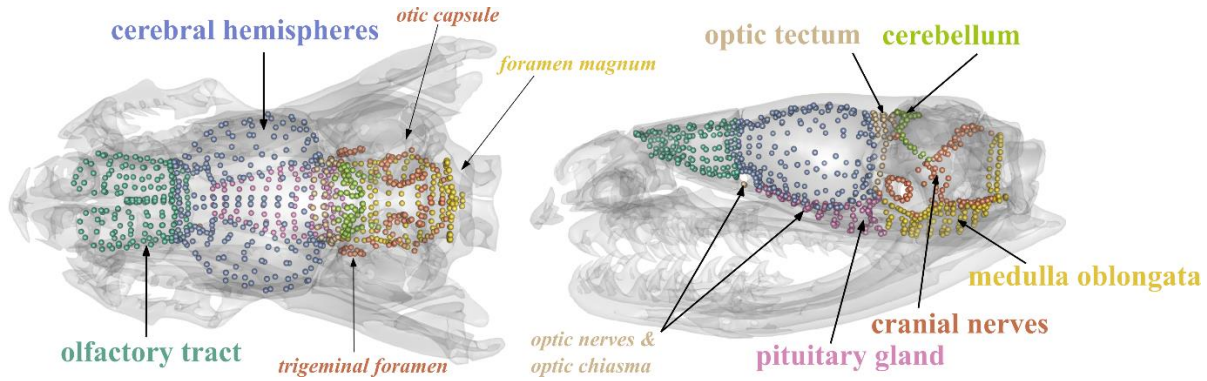
Fig. 2: Template of the endocranium of snake and its areas. *Skull of a specimen of Cantoria violacea (CAS11909) in dorsal (left) and lateral (right) view rendered partly transparent to show the 848 landmarks and semi-landmarks used. Colors correspond to brain areas (details in Supplementary Material 2b.). Important structures mentioned in this manuscript are indicated in italic and colored according to the area they belong to*

Analyses

We estimated the phylogenetic signal in the endocranium using the multivariate K-statistic (Blomberg et al. 2003; Adams 2014a) implemented in the 'geomorph' package (Adams et al. 2020) using 1000 random permutations. We tested for the phylogenetic signal in both the Procrustes coordinates and on each PC (Supplementary Material 3). We found a phylogenetic signal in the endocranium (Table 2) and in 5/9 PCs (P < 0.001), but not PC1 (P = 0.08) (Supplementary Material 3). We ran phylogenetic ANCOVA to test the effect of our predictive variables on the shape of the endocranium and its sensory areas using the function *procD.pgls* in 'geomorph' (Adams 2014b). To avoid over-parametrization of the models, each relevant factor was tested separately with size as covariate for each endocranial structure following hypotheses in Table 1. Only factors showing a significant signal were kept in the final models. We used the log-corrected centroid size as a covariate to test for evolutionary allometry. We assessed the statistical significance of the predictive variables by performing 1000 permutations of the phenotypic data at the tip of our branches. We used the same procedure for the analyses of each endocranial area. Size was removed from the ANCOVA when its distribution was not normal even after transformation, and allometry was tested separately from the predictive factors. We used the function *shape.predictor* and *mshape* from 'geomorph' to respectively extract the shapes associated with the allometry and other significant factors. Finally, we tested whether the shape of the endocranium or its sensory areas could be used to infer the sensory ecology of species for which we have little to no ecological,

248   behavioral, or phylogenetic information (e.g., elusive, or fossil species). We used a k-nearest

249   neighbour algorithm (k-NN) combined with a leave-one-out cross validation, using the function

250   *knn.cv* from 'class' package (Ripley and Venables 2020), to assess the reliability of sensory-

251   ecology classification of species based on shape data. To classify a data point of interest, this

252   pattern recognition method uses its k-nearest neighbours in terms of Euclidean distance and

253   performs a majority vote to determine which class the point belongs to. k-NN associated with a

254   cross-validation is considered to be one of the most powerful machine learning algorithms in terms

255   of predictive power and accuracy. Yet, as with many other methods, k-NN suffers from the curse

256   of dimensionality, thus we used our PC90 subsets (>90% of the shape variability for each

257   structure). The optimum k was determined for each test as follow: 1) k>1 to avoid overfitting, 2)

258   k must be inferior to the number of species in the smallest class to avoid underfitting (i.e., k<8 for

259   activity classification and k<4 for foraging habitat), 3) k with the maximum classification

260   accuracy. We choose not to correct for phylogenetic relationship to assess whether an accurate

261   classification of fossil would be possible without knowing its relationship with extant species. The

262   significant phylogenetic signals, along with our main results show that endocranial shape variation

263   is, at least partly, structured by phylogenetic relationship between species (Fig. 3). Thus, closely

264   related species are often the nearest neighbours in terms of the Euclidean distance, so if k=1, the

265   chances of the nearest neighbour being close because of phylogeny and not ecology is high.

266   Therefore, we choose k>1 to alleviate this potential issue. We used the *knn.cv* function from the

267   'class' package (Ripley and Venables 2020). All geometric morphometric, statistical analyses and

268   visualizations were performed in R version 3.4.4 (R Core Team, 2018) (R code and data available

269   in Supplementary Material), except the landmark acquisition performed in MorphoDig (Lebrun

270   2017). All statistical results are available in Table 2.

271   **Results**

272   *Phylogeny and allometry*

273   Evolutionary allometry accounts for 34% of the overall variability and respectively 40% and 49%

274   of the PC90 and mPC subsets (Table 2). These results are illustrated in Figure 3, in which size

275   drives the variation along PC1, which accounts for 45.3% of the overall shape variability. Larger

276   species gather on the negative side of the axis (PC1-), and smaller species on the positive side

277 (PC1+). Larger endocrania are characterized by antero-posteriorly elongation while small

278 endocrania are short, bulky, and laterally expanded (PC1+ and PC1- shapes in Fig. 3 and Fig. 4).

279 The variation along PC2, which accounts for 17.8% of the variability, is driven by phylogenetic

280 differences between smaller species: on the positive side (PC2+) gather the Homalopsidae,

281 Elapidae and Acrochordidae, while the Colubridae and the only Viperidae occupy the negative

282 side of PC2 (PC2-) (Fig. 3). All endocranial areas showed a significant phylogenetic signal (Kmult

283 [0.32-0.42]; P < 0.015) (Table 2). Allometries were present to different extents in all the

284 endocranial areas, except the cerebellum (Table 2) for which size was removed from the

285 subsequent analyses. The allometric trends for the individual areas follow the allometric pattern

286 described in the whole endocranium but some areas vary more (e.g., pituitary gland) or less (e.g.,

287 cranial nerves) depending on their R^2 coefficient (Fig. 4). Globally, the rear part of the

288 endocranium, where the cranial nerves insert in the brainstem, shows less allometry, especially the

289 foramen magnum (Fig. 4).

290 ***Table 2:*** *Summary of statistics: phylogenetic signal (K, P-value), number of principal components*
291 *that carry more than 90% of the variability (PC90), number of meaningful PCs (mPC), results*
292 *from the phylogenetic ANCOVAs (D-PGLS) tested on the Procrustes coordinates, 90% of the*
293 *variability and the meaningful PC based on 1000 permutations. For more clarity, only the*
294 *significant results are indicated.*

Region	Physignal	PC90	mPC	Factor	D-PGLS coordinates R	F	P	D-PGLS PC90 R	F	P	D-PGLS mPC R	F	P
Endocranium	**K= 0.38 P<0.001**	1-9	2	Size	0.34	18.95	0.001	0.4	23.97	0.001	0.49	34.47	0.001
				Activity	0.07	2.11	0.041						
Olfactory tract	**K= 0.35 P<0.001**	1-7	1	Size	0.25	13.22	0.001	0.29	15.93	0.001	0.42	30.78	0.001
				Activity	0.13	3.39	0.002	0.13	3.59	0.003	0.14	5.13	0.013
Optic tectum and nerves	**K=0.45 P<0.001**	1-5	1	Size	0.15	6.52	0.001	0.17	7.72	0.001	0.15	6.8	0.019
				Activity	0.13	2.94	0.02	0.14	3.25	0.025			
Cerebellum	**K=0.42 P<0.001**	1-7	0	Foraging Habitat	0.13	2.58	0.034	0.013	2.47	0.05			
Cerebral hemispheres	**K=0.43 P= 0.002**	1-5	1-2	Size	0.18	8.26	0.001	0.21	9.43	0.001	0.23	10.97	0.001
Cranial nerves	**K=0.41 P<0.001**	1-13	1	Size	0.08	3.32	0.013	0.09	3.74	0.013	0.18	8.16	0.017
Pituitary gland	**K=0.32 P=0.015**	1-5	1	Size	0.39	21.65	0.001	0.43	25.9	0.001	0.52	36.85	0.001
Medulla oblongata	**K=0.34 P=0.004**	1-9	1	Size	0.20	8.72	0.001	0.23	10.41	0.001	0.49	33.53	0.001

14
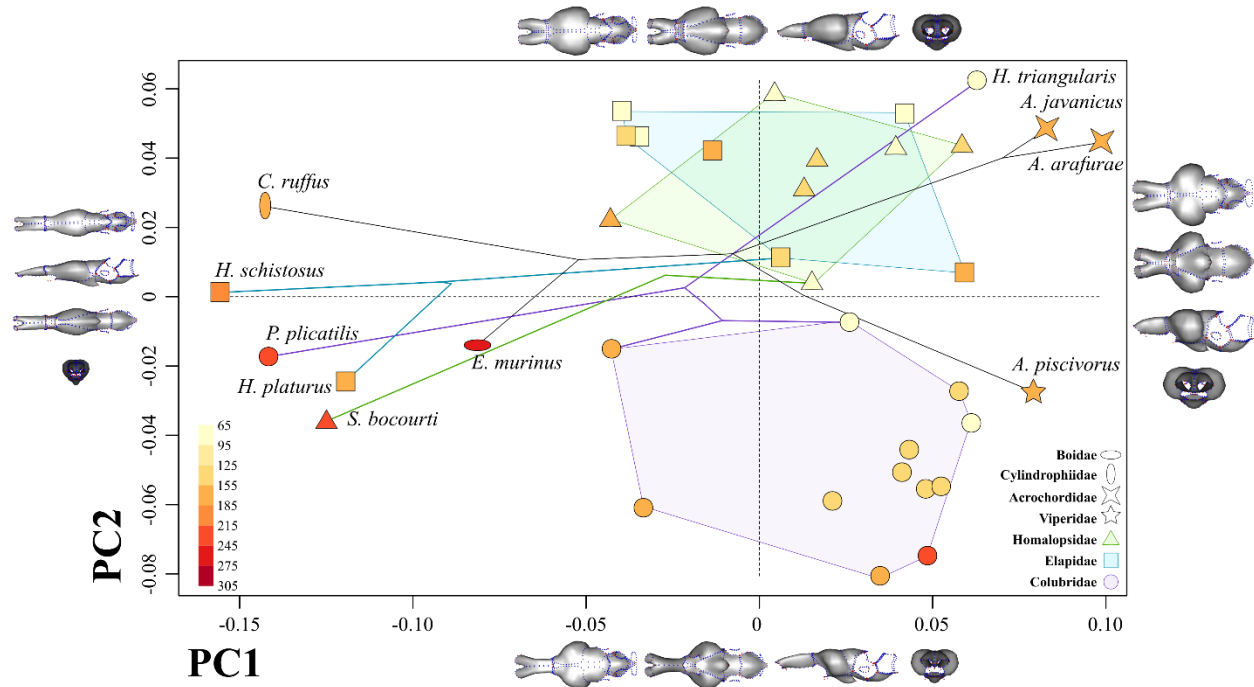
Fig. 3: Main axes of shape variation in the endocranium. *Scatter plot of the principal components one and two (PC1 & PC2) representing respectively 45.3% and 17.8% of the endocranial shape variance among the 36 snake species. Each dot represents one species, the symbols correspond to the species' family (legend bottom right corner) and the color corresponds to the centroid size of their endocranium (color scale in mm bottom-left corner). Colored polygons correspond to species grouping with their family and the colored lines indicate the phylogenetic link between outlier species and the rest of their family, black lines show the link between families with only 1 or 2 species. These links were generated using the function* phylomorphospace *in 'phytools'* (Revell 2012). *On each PC extreme are positioned dorsal, lateral, ventral, and frontal views of the corresponding reconstructed endocranium*
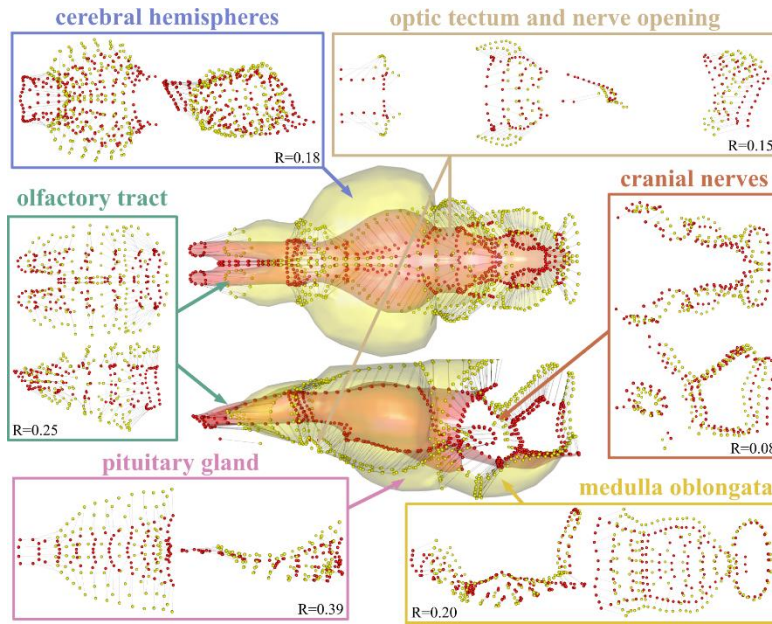
Fig. 4: Evolutionary allometry in the endocranium and its areas. *Yellow: smaller species, red: larger species. Central position: allometry in the whole endocranium. Each panel shows the allometric shape variation for each area. The R coefficients are indicated in each panel. Individual patterns of allometry for each area follows the general allometry pattern observed in the whole endocranium*

Activity period

The activity period is also significantly related to the endocranium shape but in smaller proportion than size, and it is not significant in the subsets (Table 2). The main shape variations between groups are concentrated in the areas responsible for olfaction and vision (Fig. 5b.). Coherently, the activity period is significantly related to the shape of the olfactory and optic tracts, their 90% variability subsets and the meaningful PC of the olfactory tract (i.e., PC1) (Table 2). Diurnal species have more elongated and bifurcated olfactory bulbs and their whole olfactory tract is the slenderest (Fig. 5c.), they also have the widest and longest, posteriorly extended, optic tract (Fig. 5e-f.). The endocranium of cathemeral species has an intermediate profile but shows less difference with the nocturnal species (Fig. 5b.). Cathemeral species have the shortest bifurcation of the olfactory bulbs (Fig. 5c-d.). Their optic tectum is of intermediate proportion compared to the diurnal and nocturnal species, with shorter distances with diurnal species in the middle part of the olfactory tract, while showing less variation in the most distal parts when compared to the nocturnal group (Fig. 5f.) but the anterior part of the optic tract (i.e., optic nerve opening) is the narrowest (Fig. 5e.). Nocturnal species have the shortest and bulkiest olfactory tract, but the

16

327   septum is slightly more posteriorly positioned than the cathemeral species (Fig. 5c-d.). The

328   nocturnal species have a thin and rather slender optic tract (Fig. 5e.).

329   *Foraging habitat*

330   The shape of the cerebellum and its 90% variability subset are significantly associated with

331   the foraging habitat of species (Table 2). The cerebellum of aquatic predators is more anteriorly

332   and dorsally expanded but more laterally compressed, whereas the mainly terrestrial foragers have

333   a more laterally expanded cerebellum on its anterior part, but its posterior part is more elongated

334   and slenderer, while the semi-aquatic foragers show an intermediate shape (Fig. 5 g-f.).
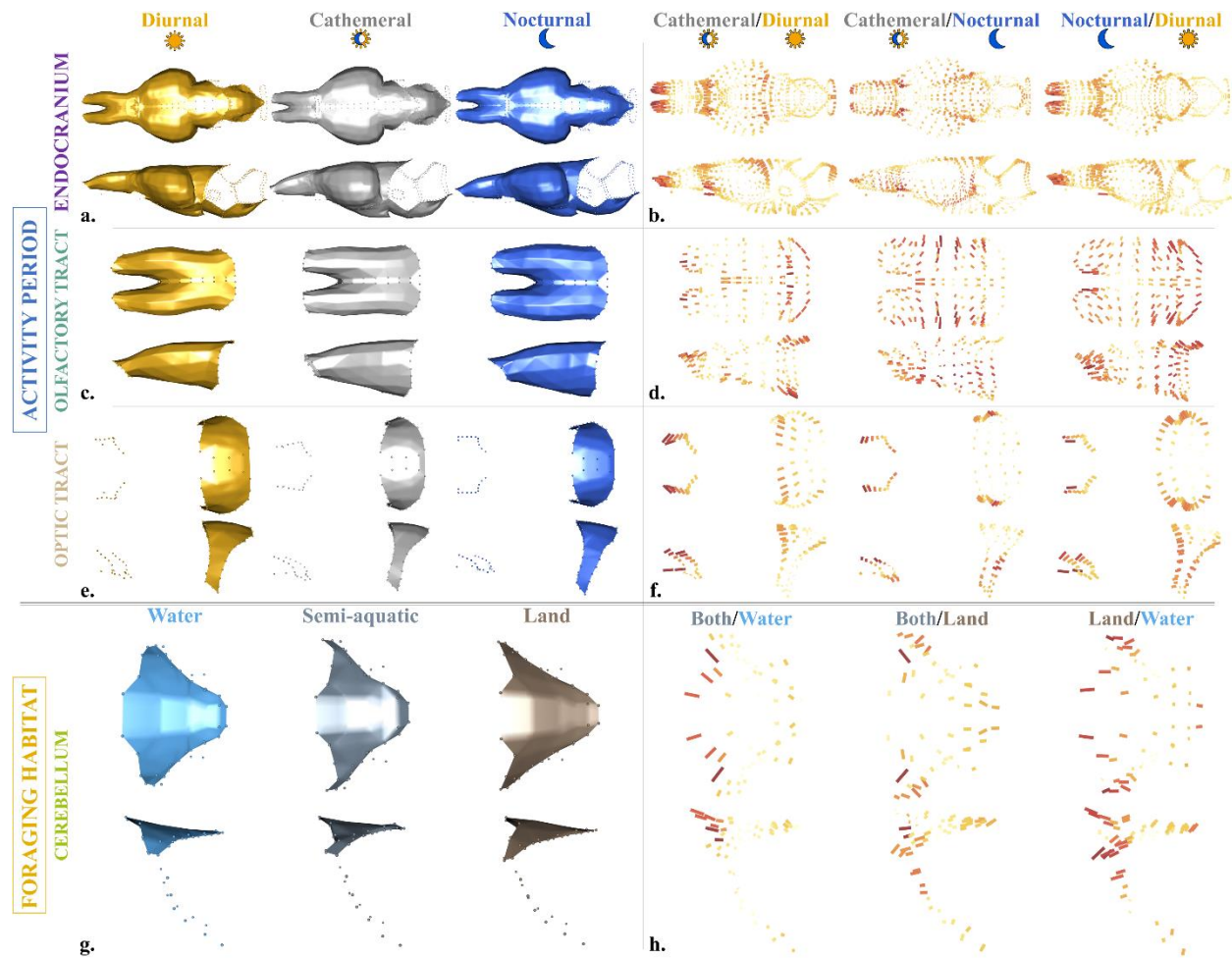
336   ***Fig. 5: Summary of the morphological variations of the endocranial structures related to the***

337   ***activity period of species (a-f) or foraging habitat (g-h) of species. a, c, e, g:*** *Mean shapes for*

338   *each group in dorsal (top) and lateral (bottom) views.* ***b, d, f, h:*** *Pairwise shape differences*

339   *between groups represented by distance vectors. Vector size and color depending on the distance*

340 *between two corresponding landmarks, from short, pale yellow to long, dark red. Color palette*
341 *generated using the 'scico' package* (Pedersen and Crameri 2020)

342   *Reclassification in ecological groups*

343 The reclassification accuracy of the activity period of species based on their olfactory tract and
344 optic tract shape is respectively 52.8% and 63.9% (Table 3). The best reclassifications for the
345 activity period are the cathemeral and nocturnal species based on their optic tract, but the diurnal
346 species are always misclassified (Table 3). For both areas, diurnal species are mostly misclassified
347 in the cathemeral group (62.5-75%), but little to no cathemeral species are misclassified as diurnal
348 (0-8%). We did not use the k-NN algorithm here as it cannot be performed on the Procrustes
349 coordinates and as our PC subsets for the endocranium did not show a significant signal for the
350 activity period. Foraging habitat reclassification accuracy based on cerebellar shape is also low,
351 with no correct classification of the terrestrial and semi-aquatic foragers but a high score for
352 aquatic predators (Table 3).

353 **Table 3:** *Reclassification accuracy of the ecology of species based on 90% of the morphological*
354 *variability of each structure (PC90) using a k-NN algorithm. Below the name of the structure are*
355 *indicated k (the number of considered neighbors) and the overall reclassification accuracy. Bold*
356 *indicates the percentages of correct reclassification per group.*

ACTIVITY PERIOD						FORAGING HABITAT			
OLFACTORY TRACT			OPTIC TRACT			CEREBELLUM			
k=5 52.8%			k=4 63.9%			k=4 58.3%			
☀	⚙	☾	☀	☀	☾	Water	Both	Land	
12.5	75	12.5	**0**	62.5	37.5	**91**	4.5	4.5	Water
8	**54**	38	0	**85**	15	89	**0**	11	Both
13	13	**73**	7	13	**80**	25	75	**0**	Land

357 **Discussion**

358   *Size, Phylogeny and Endocranium*

359 The repartition of species along the main axes of shape variation (Fig. 3) can be described by three
360 clusters: 1) large species from different snake families, 2) the Elapidae and Homalopsidae and 3)

361 the Colubridae. The main driver of endocranial shape variation is size which explains about 34%
362 of the overall variability, and half the variation of the two first components, with the minima of
363 PC1 and PC2 being driven by larger species (Fig. 3). Head dimensions are highly correlated with
364 the centroid size of the endocranium (Supplementary Material 4): species with larger heads have
365 a more elongated and slenderer endocranium, while small-headed snakes have a shorter and
366 bulkier endocranium. This pattern of globular shape and lateral extension in small species is also
367 consistent with mammals ("squirrels": Bertrand *et al.*, 2019, marsupials (Weisbecker et al. 2021),
368 and birds: Kawabe et al., 2013; Marugán-Lobón et al., 2016). The shape difference provides
369 smaller species with a relative endocranial volume almost 4 times greater than the larger species,
370 when they are at the same scale (i.e., centroid size=1). Such a difference in relative volume will
371 certainly provide an advantage for the encephalization of species with small heads, and therefore
372 potentially improve their cognitive abilities (Iwaniuk 2017). Additionally, a more globular shape
373 allows the reduction in length of the neuronal connections which has been demonstrated to increase
374 information processing speed (Sepulcre et al. 2010; Balanoff and Bever 2017). Some of our
375 preliminary results indicate that the globular shape of the small species is similar to the shape of
376 the endocranium of juvenile specimens from both small and large species of snakes. More data are
377 needed to confirm the generality of these observations, but it seems that the evolutionary allometric
378 variation originates from a retention of juvenile features (i.e., paedomorphism) in adult specimens
379 of small species, while larger species develop an elongated endocranium.

380   Phylogenetic relationships also contribute to the morphology of the endocranium but to a
381 lesser extent than size. Except for the larger species, the other species tend to cluster by family
382 along PC2, forming two main clusters: a cluster grouping the Elapidae, Homalopsidae and
383 Acrochordidae (PC2+), and another with the Colubridae (PC2-) (Fig. 3). Colubrids appear to have
384 followed their own evolutionary path regarding endocranial shape. They show the largest
385 respective size of the optic tectum compared to other shapes (Fig. 3) which is not surprising as
386 they seems to heavily rely on visual cues to detect prey (Franz 1977; Schaeffel and de Queiroz
387 1990; Alfaro 2002). Some species have even developed accommodation mechanisms to adapt their
388 visual acuity to both air and water media (Schaeffel and de Queiroz 1990; Schaeffel and Mathis
389 1991). Elapid, Homalopsid and Acrochordid snakes also use visual cues but these are not sufficient
390 to elicit a strike and are usually coupled with either mechanical (i.e. pressure variation or tactile)
391 or chemical cues (Kropach 1975; Voris et al. 1978; Heatwole 1999; Shine et al. 2004b; Vincent et

392   al. 2005; Catania et al. 2010). Evidence shows that the ancestral state of the visual system of snake

393   was adapted to low-light conditions probably associated with a nocturnal lifestyle (Schott et al.

394   2018). This is corroborated in our phylogeny with the nocturnal species representing more basal

395   lineages, while the more distal taxa (i.e., colubrids) tend to be more cathemeral or diurnal. In

396   addition, previous studies demonstrated genetic and molecular specializations of their visual

397   system (Simões et al. 2016; Schott et al. 2018). Colubrids are also characterized by a long and

398   fused olfactory tract that connects to medially expended cerebral hemispheres through a bulbous

399   region. Both structures are involved in transmitting and processing odorant cues, which are crucial

400   in prey-predator interactions and social behaviors (Halpern and Kubie 1984). While none of the

401   feeding-related factors shows any significant relationship with the global shape of the

402   endocranium, the specific shape observed in Colubrids could be explained by other activities such

403   social behaviors (Skinner and Miller 2020).

404   The large shape variation in the endocranium that we report here is not fully explained by size

405   or sensory ecology, suggesting that other behavioral or cognitive abilities may also be influencing

406   endocranium morphology. Although of great interest to us, it is currently impossible to draw a

407   direct link between endocranial shape and the evolution of elaborated behaviors in snakes, due to

408   the scarcity of data on snakes' cognitive abilities, and the phylogenetic bias of published studies

409   toward colubrids and viperids. We encourage further work to explore this potential, once it

410   becomes possible to better characterize and quantifying snake behavior in a broad comparative

411   context. It should then be able to draw stronger links between behavior and endocranium

412   morphology, and make reliable inferences not only about the ecology, but also the cognitive

413   abilities of extinct species based on their endocasts.

414   *Activity pattern, Endocranium, Olfactory and Optic tracts*

415   Although the shape of the endocranium is significantly related to the activity pattern of species,

416   most of this variation is confined to the sensory areas dedicated to olfaction and vision. Diurnal

417   and nocturnal species are the most different, while cathemeral species share similarities with both

418   groups but are overall closer to the nocturnal species. In our predictions, we proposed two

419   alternative strategies for species living in low light environments, either an enlargement of the

420   visual system or a reduction accompanied by a compensation with another sense, such as olfaction.

421　Our results demonstrate that the optic tectum in nocturnal species is slightly reduced compared to

422　diurnal species, while their olfactory tract is shorter but stouter. A similar pattern was demonstrated

423　in fish in which the most interspecifically variable areas were associated with vision, olfaction,

424　and taste (North American Shiners), and some scotopic species exhibit an enlargement of the

425　olfactory bulbs (e.g. benthic sharks, nocturnal eel-like *Calamoichthys*) (Kotrschal et al. 1998). We

426　also found a widening of the optic tectum and a shortening of the distance between nerve entry

427　and optic tectum in diurnal snake species compared to the other two groups. The hypothesis of

428　specialization of the optic tract shape in diurnal species is in accordance with previous studies on

429　genetic and molecular specialization of vision in this group (Simões et al. 2016; Schott et al. 2018).

430　As previously mentioned, it seems the visual system of snakes was originally suited for a scotopic

431　environment (Schott et al. 2018) which might explain the lack of significant difference between

432　cathemeral and nocturnal species, while diurnal species developed a more derived visual system

433　(e.g. visual adaptation in diurnal and aquatic species: Hibbard & Lavergne, 1972; Schaeffel & de

434　Queiroz, 1990; Da Silva *et al.*, 2017).

435　　　*Foraging habitat and Cerebellum*

436　In non-mammalian vertebrates, the cerebellum is involved in locomotor abilities such as

437　coordination of movements or agility, and is also the regulatory center for sensory inputs, and

438　probably involved in some higher cognitive functions such as memory and emotions (Yopak et al.

439　2017). A comparative study in squamates demonstrated a link between locomotor mode and the

440　cerebellum shape and organization (Macrì et al. 2019). Yet, they did not test for the habitat and

441　considered the 11 snake species they tested as "multi-habitat". Given the amount of overlap they

442　highlighted in the shape of the different brain structures in snakes, and considering they defined

443　the locomotor mode of species partly based on their "habitat use", it seemed coherent and

444　complementary to test for this parameter in our study, especially as species in our dataset mainly

445　use one type of locomotion on both land and under water (i.e., undulatory movements). However,

446　moving on land or under water requires different locomotor abilities and sensory inputs which is

447　reflected in the cerebellum. Foraging habitat contributes only partly to the shape of the cerebellum

448　(Table 2). It would be interesting to complete our results with more anatomical and histological

449　data, as in Macrì *et al.* (2019), focusing on aquatic and semi-aquatic species. We should then be

450　able to draw more general conclusions about the contribution of locomotion and habitat in driving

451   cerebellar shape in snakes. It should also be noted that the habitat in which a species spends most

452   of its time (i.e., its main habitat) is not statistically significant in our study while the foraging

453   habitat is, suggesting that the predator-prey interaction imposes more constraints on the sensory

454   adaptation of species. More precise definitions of "habitat use" in combination with locomotion

455   purposes (e.g., predation, thermoregulation, reproduction) should be considered as they probably

456   affect the brain differently and might allow more refined conclusion on the relationship between

457   the sensory ecology of species and their brain morphology.

458   *Can endocranium shape predict sensory ecology in snakes?*

459   One of the goals of this study was to determine if we could make accurate inferences on

460   the sensory ecology of species based on the shape of the endocranium and/or its areas. We had

461   three candidate structures, namely the optic tract and olfactory tract for the activity period and the

462   cerebellum for the foraging habitat. We chose a method that is powerful and rigorous but cannot

463   be applied to Procrustes coordinates which forced us to exclude some structures for which subsets

464   revealed they were not significantly associated with sensory ecology (i.e., endocranium). Overall,

465   we found that accurately predicting the activity pattern of species based on the shape of their visual

466   or olfactory related areas, without a priori knowledge on their phylogenetic relationship was not

467   possible. The low reclassification accuracy based on the olfactory tract could be due to the

468   dominance of allometry (Table 2). These results could be related to the imbalance of the groups in

469   our dataset (e.g., the small species sample, where some ecological groups were only represented

470   by 4 species), or it might indicate that, overall, inferring the sensory ecology of species without

471   knowing their phylogenetic relationships with other species is virtually impossible based on

472   ordinate data. These hypotheses should be tested on a broader range of species and ecologies. In

473   addition, using Procrustes coordinates rather than principal component could lead to better

474   classifications of sensory ecology as the mathematical organization of the variance resulting for a

475   PCA may hide biologically relevant information.

Conclusion

477   The aim of this study was to investigate the relationship between the sensory ecology of snakes

478   and the shape of the endocranium and its different sensory-related areas. Our results align with

479   those of previous studies on other vertebrates, but we also identify specificities concerning snakes.

22

First, size is a major component of shape disparity in the entire endocranium, and especially for the olfactory bulbs and cerebral hemispheres. Phylogeny is another key component influencing endocranial shape, with a major divergence of colubrids from the "basal" families. Finally, the shape of the endocranium, especially the olfactory and optic tract, is related to the activity period, but it does not accurately predict the sensory ecology of species without prior knowledge of their phylogeny. The shape of the cerebellum is also related to the foraging habitat. Overall, our study demonstrates that the use of shape information brings novel insights into the sensory adaptations of snakes. The study of the morphology of brain related structures is also expected to be complementary to volumetric information and histological studies, and will further characterize the relationship between the endocranium, the brain, and the sensory ecology of species. However, endocranial morphology alone cannot be used to infer the sensory ecology of species.

We hope our results will encourage further work on brain anatomy, morphology, and behavior of snakes, as these animals remain largely underrepresented in neurobiological studies, as strikingly demonstrated in the most recent and extensive book on the evolution of nervous systems in vertebrates (Kaas 2017). The ecological and behavioral diversity of snakes, along with their underestimated cognitive abilities associated and more basic brain structure, makes them a valuable model to understand the evolution of more complex brains as suggested in (Naumann et al. 2015).

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