The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2016, **119**, 106–116. With 4 figures.

Intraspecific morphological variation of the middle ear in the European badger, *Meles meles* (Carnivora: Mustelidae)

DAVID ROZEN-RECHELS[1,2]*, STÉPHANE PEIGNÉ[1], DAMIEN GERMAIN[1] and SANDRINE LADEVÈZE[1]

[1]*CR2P – UMR 7207 CNRS, MNHN, Univ Paris 06, 57 rue Cuvier, CP 38, F-75005, Paris, France*
[2]*École normale supérieure, Formation Interuniversitaire de Biologie, 46 rue d'Ulm, FR-75230, Paris Cedex 05, France*

Received 19 January 2016; revised 15 February 2016; accepted for publication 15 February 2016

Many studies have been conducted about the information contained in the anatomy of the mammalian middle ear. Most of these only use a few specimens. Thus we aim to provide a quantitative analysis of the intraspecific and interspecific variations of the middle ear, focusing on the auditory bulla. For that purpose, we focused on the mustelids, as a quite generalist taxon and, more specifically, on the European badger, *Meles meles*. Our study includes two types of statistical methods. We first compared the mean of a subjectively chosen measure between individuals of the same species and between individuals of different species. We then used a multidimensional scaling procedure to cluster individuals according to different measures. We conclude that the middle ear varies effectively less intraspecifically than interspecifically. However, we think that the few anatomical parameters to measure in the auditory bulla involve using more specimens or focusing on geometric morphometrics in studies focusing on middle ear. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **119**, 106–116.

KEYWORDS: 3D-segmentation – auditory bulla – interspecific variation – Mammalia – multidimensional scaling.

INTRODUCTION

Perception of the outside world by organisms is highly diversified and constrained by natural selection (Wever, 1974). Hearing is the process through which the ears detect and perceive sounds (Fay, 1994). Ears are crucial for hearing and for controlling a sense of position and balance, thanks to their tripartite organisation in the outer, middle, and inner ears through which the sound waves are collected and propagated to be translated into electrical signals sent to the brain (Davis *et al.*, 1934). These different parts of the ear have been widely studied in evolutionary biology, either to address the evolutionary implications of the form-function relationships or to evaluate the relationships between environmental parameters and auditory capabilities (e.g. Rosowski, 1994; Manley, Popper & Fay, 2004; Gridi-Papp & Narins, 2009; Coleman & Colbert, 2010). Our contribution focuses on the middle ear, the morphology of which has been interpreted for decades as a proxy for hearing capabilities and specialization in mammals (Zavattari, 1938; Petter, 1953; Legouix, Petter & Wisner, 1954; Webster, 1962, 1966; Lay, 1972; Fleischer, 1978). Many recent publications present comparative studies about the structure and functions of the middle ear (e.g. Huang, Rosowski & Peake, 2000; Takechi & Kuratani, 2010; Salih *et al.*, 2012; Wible & Spaulding, 2012) or studies addressing the relationships between the structure of the middle ear and the environment (e.g. Huang *et al.*, 2002; Schleich & Vassallo, 2003; Argyle & Mason, 2008). A remarkable example is the existence of a large auditory bulla in rodents (Webster, Ackermann & Longa, 1968; Lay, 1972) and felids (Huang *et al.*, 2002) living in open and arid habitats such as deserts. This structure is an adaptation to low-frequency sounds according to Webster *et al.* (1968) and

*Corresponding author. E-mail: david.rozen@wanadoo.fr

Lay (1972). Most of those studies, however, focus on interspecific variation of a trait. None of them considered the range of intraspecific variation as a potential source of error. Moreover, the low number of specimens does not exclude the potential of considering an outlier as a model for a species. Most authors used around five specimens per species for their focus taxa (e.g. Mason, 2004; Argyle & Mason, 2008). Some others only used one or two specimens (Huang *et al.*, 1996, 2000; Wible & Spaulding, 2012) or did not even state clearly how many specimens they used (Nummela, 1995). It is still unclear whether the middle ear is a structure with a great amount of anatomical variability or not. Rosowski (1994) highlighted the high intraspecific variation of the volume of the bulla in humans. If this is more generally the case, most of the previously cited studies and many others have to be conducted again, using more specimens.

Studies on the middle ear often focus on highly specialized taxa (e.g. the sand cat living in deserts in Huang *et al.*, 2002 or subterranean rodents in Schleich & Busch, 2004) or work at a high phylogenetic scale, such as Mammalia (see Nummela, 1995). In ecology, specialists use a narrow range of resources (e.g. Terraube *et al.*, 2011). By analogy, we suppose we would find less variable structures in a specialist taxon than in a generalist one. To determine the range of intraspecific variation in such structures, we chose a model taxon that is as generalist as possible. The European badger *Meles meles* (Linnaeus, 1758) (Melinae, Mustelidae, Carnivora, Mammalia) is a generalist in the context of our study because it is able to use diverse habitats to forage (see Elmeros, Madsen & Prang, 2005), and hunts at the surface but also digs burrows. According to the latest taxonomic status, its distribution goes from the British Isles to the Volga River in Eastern Europe (Abramov & Puzachenko, 2013). Moreover, in Europe, its craniometrical variability is very low (Abramov & Puzachenko, 2006), even though two subpopulations can be distinguished in continental Europe and Scandinavia (Abramov, Puzachenko & Wiig, 2009).

In this study, we aimed at characterizing the pattern of variation of some structures of the middle ear, especially the auditory bulla volume, in a chosen clade of mammals. In order to characterize the intraspecific variation of the middle-ear anatomy of *Meles meles*, we compared it to the interspecific variation of the same traits with close species [namely five additional mustelid species showing a diversity of behaviours and phenotypes (see Table 1)]. Our aim was to test whether the quantitative variation of a trait between two individuals of *Meles meles* was significantly lower than that between one European badger and one specimen of any other species. Our goal was to find out whether the middle ear shows little intraspecific variation, allowing it to be compared interspecifically using a few specimens and also if it can be discriminant enough between close species.

MATERIAL AND METHODS

The study was based on 33 skulls of mustelids with 21 skulls of the European badger *Meles meles* (Linnaeus, 1758), three skulls of the European otter *Lutra lutra* (Linnaeus, 1758), three skulls of the hog badger *Arctonyx collaris* (F. G. Cuvier, 1825), two skulls of the wolverine *Gulo gulo* (Linnaeus, 1758), two skulls of the honey badger *Mellivora capensis* (Schreber, 1776) and two skulls of the American badger *Taxidea taxus* (Schreber, 1777). All specimens were from the Zoology Collection (Mammifères, ZM) of the Muséum national d'Histoire naturelle (MNHN), Paris, France (see references in Table 1).

Computed tomography (CT) scanning was conducted at the X-ray Tomography Imagery Platform AST-RX of the MNHN, using a GE Sensing and Inspection Technologies phoenix|x-ray v|tome|x L240-180 CT scanner. The scan parameters are described in Supporting Information (Table S1) except for exposure time, which is consistently 333 ms. Three thousand projections over 360° were performed, with three averaged images per projection and one skipped image before each projection. The data were reconstructed using phoenix datos|×1 2.0 reconstruction software, and then exported into a 16-bit TIFF image stack.

From this CT acquisition, we constructed a 3D segmentation model of the middle ear. We considered here the malleus and incus (the stapes bone was sometimes absent and more difficult to model) and the cavity of the auditory bulla. We defined the auditory bulla as the cavity between the pot-shaped part of the ectotympanic bone where the tympanic membrane is fixed and the oval window (*fenestra vestibuli*), minus four fossae, interpreted as follows: the epitympanic recess (*recessus epitympanicus*), the tensor tympani (*musculus tensor tympani*) fossa, the geniculate ganglion (*ganglion geniculi*) space, and one last space anterolateral to the geniculate ganglion space, more on the inside of the skull, that we could not identify. These structures are presented in Figure 1A. We also modelled the entire skull in order to measure different external traits (Fig. 1B).

Post-processing was performed at the Paleontology Imaging Unit of the MNHN Département Histoire de la Terre/CNRS UMR 7207. In order to optimize the post-processing, the stack was cropped, corrected (level balance, brightness/contrast), and converted to

Table 1. Specimens chosen for the study, their sampling location, and the lifestyle of the species

Species	Lifestyle	Habitat	Specimens (MNHN-*)	Location
Arctonyx collaris	Semi-fossorial (1)	Close (2)	ZM-AC-1877-704	NA
			ZM-AC-1961-186	NA
			ZM-MO-1962-1638	China
Gulo gulo	Terrestrial (3)	Mixed (4)	ZM-AC-1967-54	NA
			ZM-2005-853	Canada
Lutra lutra	Aquatic (3)	Aquatic (3)	ZM-MO-1962-1738	France
			ZM-2005-597	France
			ZM-2005-598	Tunisie
Meles meles	Semi-fossorial (3)	Mixed (4)	ZM-MO-1916-17	NA
			ZM-MO-1937-1256	France
			ZM-MO-1948-514	NA
			ZM-MO-1962-1009	NA
			ZM-MO-1962-1727	France
			ZM-MO-1962-1728	France
			ZM-MO-1962-1729	France
			ZM-MO-1962-1730	France
			ZM-MO-1962-1731	France
			ZM-MO-1962-1732	France
			ZM-MO-1962-1733	France
			ZM-MO-1962-1734	France
			ZM-MO-1962-1735	France
			ZM-MO-1982-172	France
			ZM-MO-1985-2020	France
			ZM-AC-1987-28	NA
			ZM-MO-1991-604	France
			ZM-MO-1996-2167	France
			ZM-MO-1996-2439	France
			ZM-MO-1998-1247	France
			ZM-2005-231	NA
Mellivora capensis	Semi-fossorial (3)	Open (5)	ZM-MO-1893-6	Tanzania
			ZM-MO-1995-3150	Mauritania
Taxidea taxus	Semi-fossorial (3)	Open (4)	ZM-AC-1895-417	United-States of America
			ZM-MO-1927-2367	Mexico

(1), Rose *et al.*, 2014; (2), Rabinowitz & Walker (1991); (3), Fabre *et al.* (2015); (4), Gittleman & Harvey (1982); (5), Begg, Begg & Abramov (2008); NA, non-attributed.

8-bit images with ImageJ software (Abràmoff, Magalhães & Ram, 2004) for final image stacks of the bullae described in the Supporting Information (Table S2). The processed image stack of the skull was obtained after a binning, which divided the number of voxels by 2, and was then corrected and converted in 8 bits. Mimics1 v.17.00 (Materialise, Leuven, Belgium) was used for the 3D-modelling (segmentation and 3D-object rendering).

From these models the following parameters were measured: skull length L_s, skull width W_s, skull height H_s, interbullae spacing B_b, bulla length L_b, bulla width W_b, bulla volume V_b, malleus volume V_m, incus volume V_i, oval window length L_o, oval window width W_o, tympanic membrane length L_t, tympanic membrane width W_t. All traits were measured three times and we considered the mean of those measures to reduce the error range. We also calculated other variables from these data: skull volume V_s, oval window surface S_o, tympanic membrane surface S_t, and the ratio of those two surfaces R (see Figs 1 and 2; Table 2, for more informations).

We chose to focus on the volume of the auditory bulla as the main trait of our study, as it is its hypertrophy that has often been linked to arid environments (e.g. Webster & Webster, 1980; Huang *et al.*, 2002). Using linear regressions, we analysed the correlation between this volume (mean volume of the two bullae of one individual) and V_s and the sum of the malleus and incus volumes for each bulla (i.e. $V_m + V_i$).

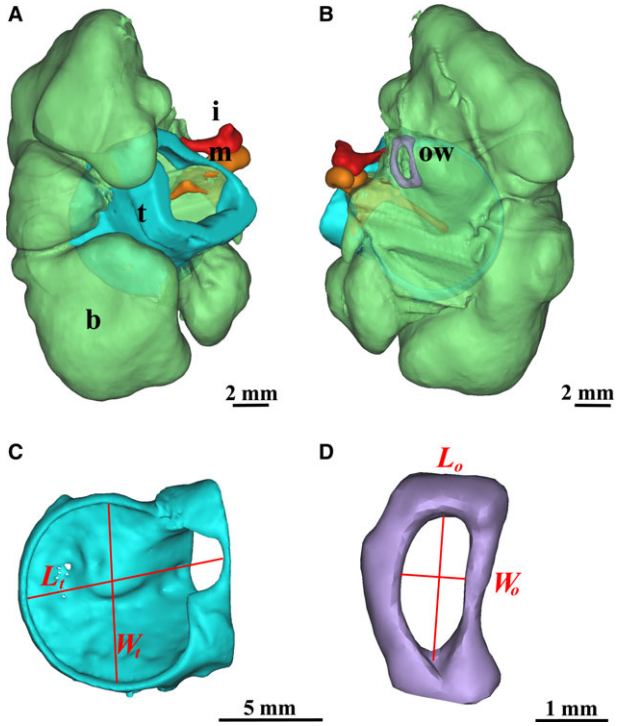

Figure 1. Three-dimensional (3D) model of the right auditory bulla of *Meles meles* (MNHN-ZM-MO-1962-1735). A, Ventral view of the auditory bulla cavity (b), with the pot-shaped part of the ectotympanic (t), the malleus (m) and the incus (i). B, Dorsal view of the same structure on which appears the bone surrounding the oval window (ow). C, Pot-shaped part of the ectotympanic and the measure of L_t and W_t. D, Bone surrounding the oval window and the measure of L_o and W_o.

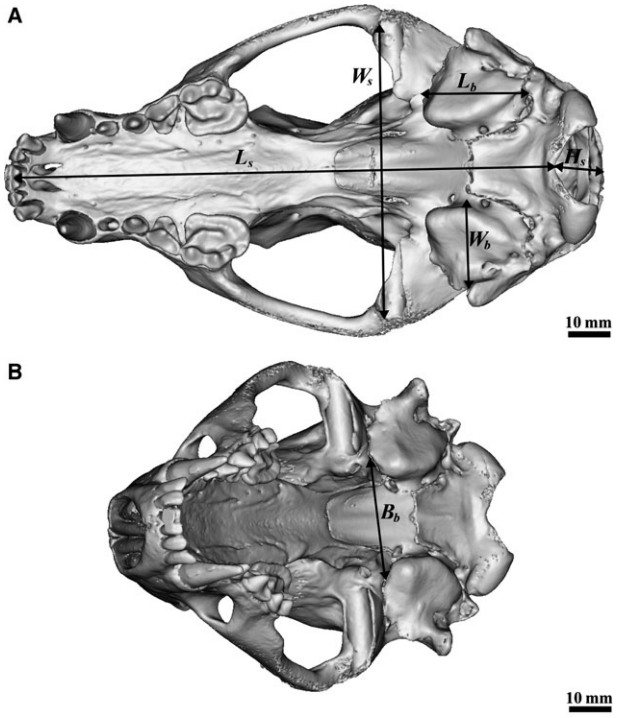

Figure 2. Three-dimensional (3D) model of the skull of *Meles meles* (MNHN-ZM-MO-1962–1735). A, Ventral view of the skull with the measure of L_b, W_b, L_s, W_s, H_s. B, ½ view between front view and ventral view in order to show the B_b measurement.

Intra-individual and the intraspecific variation

To estimate the variation of the auditory bulla between the two bullae of the same individual, we calculated the absolute difference of volume between the right bulla and the left bulla. We compared it to the absolute difference of the volume of two sets of randomly chosen bullae. We also compared the mean difference in the volume of the bulla between the left and the right sides for all species. We then only used the mean volume of the two bullae for each individual. In this analysis, as we compared sets of data of the same length, we tested the difference between the different sets of data with an analysis of variance (ANOVA) (using the *lm* and *anova* function in R (R Core Team, 2014)). In every case, we checked the Gaussian distribution of the residuals, the significance of our model compared with the null model, and the significance of the parameters. We only present the results of the parameters.

Intraspecific and the interspecific variation

To compare the intraspecific variation to the interspecific variation, we focused on the volume of the bullae relatively to the volume of the corresponding skull V_s. As the shape of the bullae changes between species, it seemed difficult to only consider the volume of the bullae to compare species. We considered it relevant to use this volume relative to the skull as quantitative translation of the size of the middle ear according to the species. We calculated all the absolute differences of the volume of the bulla relative to the volume of the entire skull between each European badger and all the absolute differences between all European badgers and any specimen of another species separately. We compared the mean of the differences between the volume of the bulla relative to the volume of the skull of a European badger with the one of a specimen from another species to the same difference between two specimens of European badger. In this analysis we first looked at the normality of our set of values using the Kolmogorov–Smirnov test. If our data were of a normal distribution, we used Student's *t*-test to compare the means. If not, we used the Wilcoxon rank test.

Table 2. Traits measured or calculated in the study and their description

Trait	Description or formula	Abbreviation
Length of the skull	Length between the prosthion and the basion	L_s
Width of the skull	Length between the two extremities of the mandibular fossa	W_s
Height of the skull	Length between the inion and basion	H_s
Bullae spacing	Length between the two exterior points of the opening of the musculotubal canal	B_b
Length of the bulla	Maximum length parallel to the prosthion-basion axis	L_b
Width of the bulla	Maximum length perpendicular to the prosthion-basion axis	W_b
Volume of the bulla	Volume estimated from the segmentation of the cavity of the auditory bulla minus four fossae: the epitympanic recess, the tensor tympani fossa, the geniculate ganglion, and one non-identified fossa	V_b
Volume of the malleus	Volume estimated from the segmentation of the malleus bone	V_m
Volume of the incus	Volume estimated from the segmentation of the incus bone	V_i
Length of the oval window	Major axis of the round window	L_o
Width of the oval window	Minor axis of the round window	W_o
Length of the tympanic membrane*	Major axis of the pot-like structure of the ectotympanic	L_t
Width of the tympanic membrane*	Minor axis of the pot-like structure of the ectotympanic	W_t
Volume of the skull	$L_s \times W_s \times H_s$ Volume of the smallest box containing the entire skull	V_s
Surface of the oval window	$\pi \times L_r \times W_r$ Surface of the ellipse described by L_o and W_o	S_o
Surface of the tympanic membrane*	$\pi \times L_t \times W_t$ Surface of the ellipse described by L_t and W_t	S_t
Ratio between the round window and the tympanic membrane*	$\frac{S_r}{S_t}$ Quantitative comparison of the oval window and the tympanic membrane	R

*We estimated the ellipse created by the pot-like part of the ectotympanic bone as being a good predictor of the tympanic membrane. We suppose that the real surface of the tympanic membrane is highly correlated to this ellipse. As the malleus moved in most skulls, we were not able to estimate it in a better way.

Interspecific variation and cluster of individuals

We conducted a multivariate analysis (James & McCulloch, 1990) to analyze the pattern of relationships among individuals in a blind way (not considering the species). In other words, we tried to blindly cluster the individuals and check if we can determine that our species are clearly separated. This implies taking into account dissimilarity matrices. We followed the methodology detailed in Abramov *et al.* (2009). The traits we used in this analysis are V_s, L_b, W_b, B_b, R, V_m, V_i, V_b (see Table 2). In order to exclude any influence of allometry, the measures were standardised according to the following equation:

$$x'_i = \frac{x_i - x_{\min}}{x_{\max} - x_{\min}}, \quad (1)$$

where x'_i is the standardized value, x_i is the measured value and x_{max} and x_{min} are respectively the maximum and the minimum value of the measurement distribution. As proposed by Abramov *et al.* (2009; p. 435), this formula 'retains the shape of the sample distribution and does not equalize variance'.

We then created two dissimilarity matrices. The first one was the Euclidean distances matrix among all the pairs of specimens. The second one was created from the matrix of Kendall's tau-b rank-order coefficients among all pairs of specimens. Defining k_{ij} as an element of this matrix, we calculated the dissimilarity matrix of coefficients $d_{ij} = \sqrt{1 - k_{ij}}$ named Kendall's coefficients. These coefficients measure how similar are the data when rank-ordered, according to different traits. It then measures for two specimens the probability to be ranked in the same order as most of the traits against the probability that they

are not. As Abramov *et al.* (2009; p. 436) stated, we have then a measure of the 'shape' of the skull as we observe 'the concordance in variation of different measurements from one specimen to another'.

The multivariate analysis we chose to use is the nonmetric multidimensional scaling (NMMDS) procedure (Shepard, 1962; Kruskal, 1964; Davison, 1983). Simply put, this procedure tries to find n points in a k-dimension space, n being the number of specimens we have, for which the distances 'correspond' to the differences between our specimens. The MDS is more robust than the principal component analysis (PCA) as it does not take into account any assumption on the relationships between variables (Abramov *et al.*, 2009). To run our MDS procedures we used the 'metaMDS' function of the *vegan* package (Oksanen *et al.*, 2014; R Core Team, 2014).

As in a PCA, the MDS produces dimensions (called axes), which places points corresponding to our specimens. To choose the best number of dimensions, we chose to follow the method described in Abramov & Puzachenko (2005), as did Abramov *et al.* (2009). We created a random dataset of the same dimensions in which each variable is of a random normal distribution of the same mean and standard deviation as the variable in the actual dataset. For the real and the random dataset we ran the MDS procedure calculating the stress value of the analysis for each dataset from one to 15 dimensions (analogous to Cattell, 1966). If the MDS procedures are conducted in the same way for the two datasets, the best number of dimensions k can be found by doing the following regression:

$$S_i = A \times S_{oi} + B + \varepsilon_i, \quad (2)$$

with S_i as the value of the stress function for the real data, S_{oi} as the value of the stress function for the random dataset, i as the number of dimension, A and B are two constants and ε_i as the error function. More details can be found in Abramov *et al.* (2009). In the following parts, the axes of the MDS based on the Euclidean distance matrix are called E1, E2, ... and the ones of the MDS based on the Kendall's correlation matrix are K1, K2, ...

In order to give an interpretation of the MDS axes, we calculated the Spearman's rank-order correlation coefficients between each variable and each axis. We chose to consider a Spearman score as relevant according to the criteria described in Abramov *et al.* (2009), i.e. when equal to or higher than |0.5|. We also calculated the value of explained variance (or the square of the multiple correlation coefficients) of each variable, allowing us to check the stochastic variability of our measurements.

From the coordinates of the points in the space defined by the two MDS dimensions, we clustered our individuals using a hierarchic classification using an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method based on the Euclidean distance between the points in the MDS axes space.

RESULTS

There was no correlation between the volume of the skull and the volume of the bulla (linear model P-value: 0.47; correlation coefficient: −0.13). The bigger the bulla, however, the bigger the ossicles, (here $V_m + V_i$; estimate of the slope of the linear relation: 0.002; linear model P-value: 0.004; correlation coefficient: 0.49). We observe the same pattern between the volume of the skull and the volume of the ossicles (estimate of the slope of the linear relation: 0.00002; linear model P-value: 0.008; correlation coefficient: 0.46).

We considered the difference between the volume of the right and the left bullae and the difference between two bullae taken randomly in the *Meles meles* dataset. These two samples are significantly different (ANOVA type II test: 1 degree of freedom, F-value = 14.144, P-value < 0.001). The absolute difference between the two bullae taken randomly (in average 150.35 mm^3, P-value < 0.001) is significantly higher (difference of −106.17 mm^3, P-value < 0.001) than between two bullae of the same skull.

The difference between the volume of the bullae relative to the volume of the skull is not normally distributed in the intraspecific case (Kolmogorov–Smirnov test: P-value = 0.02). We then performed a Wilcoxon rank test to compare the intraspecific and the interspecific variation. The results of these tests can be found in Table 3. In every case, the difference of ratio between two individuals of the same species

Table 3. Intraspecific vs. interspecific variations

Species compared with	P-value	Average intraspecific variation	Average interspecific variation
Arctonyx collaris	< 0.001	0.0006	0.003
Lutra lutra	< 0.001		0.002
Gulo gulo	< 0.001		0.001
Mellivora capensis	< 0.001		0.004
Taxidea taxus	< 0.001		0.013

For each species other than *Meles meles*, we have presented the P-value calculated using Student's t-test and comparing the mean of the volume of the bulla divided by the volume of the skull between the skulls of *Meles meles* and between the skulls of the other species and the skulls of *Meles meles*.

Table 4. Spearman correlation coefficient between each parameter and each axis of the two produced MDS and multiple correlation coefficient for each parameter (or deviation explained by the two MDS)

	MDS axes					
Trait	E1	K1	K2	K3	K4	r^2
V_b	−0.28	−0.17	−0.36	**0.68**	**0.61**	0.82
B_b	−0.17	−0.01	0.18	−0.09	0.02	0.78
L_b	**−0.64**	−0.12	**−0.64**	0.02	0.07	0.96
W_b	**−0.71**	0.40	0.46	−0.09	0.22	0.93
V_m	**−0.67**	−0.42	−0.27	−0.09	0.17	0.74
V_i	**−0.73**	0.12	−0.19	**0.60**	−0.03	0.89
V_s	−0.48	**0.64**	0.11	**0.56**	0.21	0.77
R	0.37	0.44	0.26	**0.59**	**−0.53**	0.45

In bold are presented relevant coefficients.

is significantly lower than between two specimens of two different species (one European badger and one of any other species).

The best number of dimensions for the MDS based on the Euclidean distances matrix is 1 and 4 for the other one. Following Abramov *et al.* (2009), we then have one axis describing the size variation and four for the shape variation (Table 4). The only 'size' axis is mainly explained by the dimensions of the bullae and the volume of the ossicles. The third 'shape' axis is one explained by most of the characters. All of those are describing the middle ear: volume, ratio between the oval window and the tympanic area, the skull volume and the incus volume. K3 is then highly explained by the shape of the middle ear. K1 is explained by the volume of the skull, K2 is explained by the length of the bulla, and K4 is explained by the volume of the bulla and the ratio R. Graphically, we see that the 21 badgers are quite well clustered and distinguishable from the other species. The other species are, however, not well separated between one another (Fig. 3). The volume of the skull (K1 axis in Fig. 3A) is the parameter that separates European badgers the most from the other species. Dimension E1 does not isolate this species

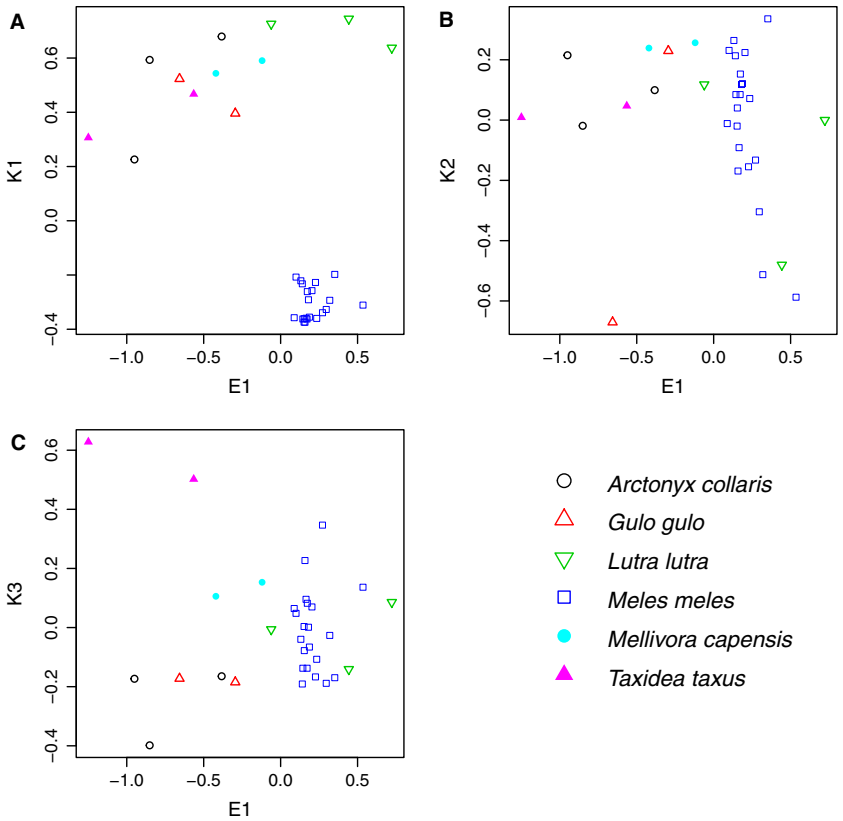

Figure 3. Graphic result of the two MDS procedures. Position of each specimen in the MDS dimensions space for E1 and the three first axes of the MDS based of the Kendall's tau-b coefficients matrix. According to Table 4, graph A was then plotting a space described by the bulla and the volume of the skull; graphs C and to a lesser extent B plotted a space only described by the bulla. Even though the skull allows a good separation of the badger from the other species, the bulla itself does not distinguish between the otter and the Europen badger.

from the otters even though it isolates it from the other species. Dimensions K2 and K3 (respectively Fig. 3B, C) alone do not isolate *Meles meles*, even though they allow us to distinguish this species from the other species associated with parameter E1.

The UPGMA procedure gives a tree with a strong significance (cophenetic correlation of 0.91). This tree (Fig. 4) has two main branches, one clustering all European badgers, the other one clustering all other species. Except for *Taxidea taxus* and *Mellivora capensis*, all specimens are mixed on this branch. The branch clustering all *Meles meles* is also closed to a branch clustering two otters, with the same root, illustrating the difficulty to isolate what we discussed regarding Figure 3.

DISCUSSION

The main preliminary result of our study was the relative symmetry of the middle ear in size in one individual. No previously cited publications took this potential source of variation into account (often averaging for left and right bulla). A significant asymmetry would have meant a difference between left and right leading to a potential misunderstanding of the interspecific variation if the size were averaged for each individual. Our results allowed us to support previous publications, even though middle ear size should be an essential parameter to check every time, essentially as we only tested our assumptions in mustelids and more specifically *Meles meles*.

We considered the volume of the bulla divided by the volume of the skull as the best parameter estimator of the size and shape of the middle ear. This parameter varies significantly less intraspecifically than interspecifically. This factor means that this structure is 'stable' enough to be addressed in evolutionary studies. In other words, an observed variation between two species is not an artefact created by the choice of the specimens but a real difference. Therefore, the choice of a few individuals per species seems relevant when studying interspecific variation.

Such interspecific comparisons might be, however, more complex if using diversified measures. The MDS procedure we used manages to discriminate European badgers from the other species. The auditory bulla discriminated *Lutra lutra* and *Meles meles* from the other species (E1 and K3 axes of the MDS). It was only the volume of the skull that discriminated the European badger (see Fig. 3A). We note that the auditory bulla measurements discriminated quite efficiently *Taxidea taxus* and *Mellivora capensis* (see Fig. 3C), which have hypertrophied bullae compared with the other species. We could then suppose that the middle ear of the latter two is specialized or that the ear of *Meles meles* is quite similar even knowing their huge difference in behaviour.

It was harder to differentiate the species that have been described by only two or three skulls, taking the entire MDS into account (see Fig. 4). We would need more characteristics to anatomically describe the middle ear. It might be difficult to find characteristics that are not already highly correlated to the ones we used, interspecifically homogeneous, and available in skeletons (see Schleich & Busch (2004) who had more characteristics but in only one species and using captured individuals).

It is worth pointing out that the volume of the bulla relative to the volume of skull is not linked to the animal's lifestyle. *Taxidea taxus* has the biggest bulla relative to the skull with a ratio of 0.017, whereas the ratio for *Meles meles* or *Arctonyx collaris* is respectively 0.0039 and 0.0012 (the smallest ratio). However, these three are semi-fossorial animals. Conversely, we observed a potential link with the habitat. *Taxidea taxus* and *Mellivora capensis* present the biggest bulla volume relative to the skull volume (0.0075 for the last one). *Taxidea taxus*' home range is most limited to the prairies in North America, living in open grasslands (Gittleman & Harvey,

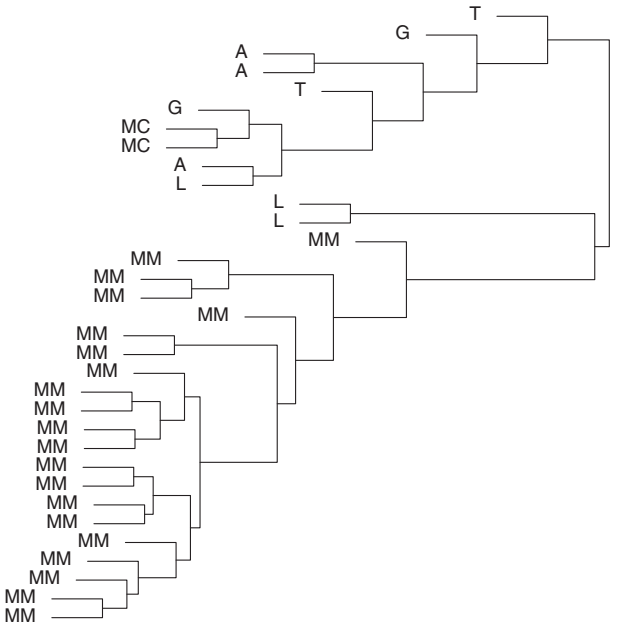

Figure 4. Tree produced by the UPGMA clustering method. A, *Arctonyx collaris.* G, *Gulo gulo.* L, *Lutra lutra.* MC, *Mellivora capensis.* MM, *Meles meles.* T, *Taxidea taxus.* The entire MDS allows us to cluster the European badger separately from the other species, however we believe there are not enough parameters to highlight the similarities between the specimens of the same species for which we only had two or three skulls.

1982). *Mellivora capensis*' case is more tricky as it lives in a huge variety of habitats (Begg, Begg & Abramov, 2008). The skulls from our samples came from individuals living in deserts, steppes and savannas (Mauritania and Tanzania). Both of these species are adapted to open habitats. However, *Arctonyx collaris*, which has the smallest bulla volume relative to the skull, lives in tropical forests (Rabinowitz & Walker, 1991). The other species that have an intermediary bulla volume live in mixed habitats (e.g. *Meles meles* and *Gulo gulo* in Gittleman & Harvey, 1982) or in a highly different type of habitat (e.g. *Lutra lutra* in rivers). This observation is a good clue that supports studies such as by Huang *et al.* (2002) interpreting hypertrophy in the middle ear as an adaptation to arid open habitats. Ear specializations to different lifestyles are more marked in the inner ear (Crumpton, Kardjilov & Asher, 2015); this situation implies that plasticity in this structure could greatly affect the behaviour of the animals.

Such a low rate of variation could be interpreted as evidence that the middle ear is under strong evolutionary constraints. Moreover, some specializations of the middle ear to extreme environments (e.g. the hypertrophy of the bulla of animals living in arid environments; Huang *et al.*, 2002) can also be found in rodents (e.g. Lay, 1972) and in Xenarthra (Squarcia, Sidorkewicj & Casanave, 2007), which inhabit desert or open spaces. This strengthens the relevance of this anatomical and functional area as an ecological or behavioural proxy. The study of such structures that are extremely conserved could give more clues to the understanding of evolutionary processes.

We did not find any other study examining this variation even though it has now been a long time since zoologists first pointed out this limitation to the study of adaptation. Cuvier (1825) already stated that we should, 'examiner jusqu'où s'étendent ces limites, recherche curieuse, fort intéressante en elle-même sous une infinité de rapports, et dont on s'est cependant bien peu occupé jusqu'ici' [look into the extensions of those limits, a curious and in many ways highly interesting research per se, about which we have not really cared for until now] in order to address claims that variability is fixed in a defined range. We likewise encourage any research project on anatomy and morphology begin with studying the variation of the traits of interest.

As the middle ear, in the way we studied it, did not seem to be a good interspecific discriminant, other ways to study its anatomy should also be investigated. Pfaff, Martin & Ruf (2015) developed a 'septal compass' to study phylogenetic relationships using the middle ear septa. We suggest such a method should be tested on other clades, even though it seems to us that the septa are different between the bullae of the same specimen in our badgers. Another possible approach is geometric morphometrics (Adams, Rohlf & Slice, 2004). Such a procedure has already been used to study intraspecific variation in the inner ear (Billet *et al.*, 2012). It is, however, harder to choose landmarks in the bulla. The surface of the bulla volume does not show any real homologous points. One could focus on using this procedure on the external surface of the bulla where foramina and bones provide more opportunity to place landmarks. We, however, believe that further studies should focus on finding a new approach to 'envelop' a surface with landmarks without a high number of homologous points. This kind of method could then be a highly efficient way to study the shape of the mammalian middle ear.

ACKNOWLEDGEMENTS

We thank the anonymous reviewers who helped us improve the manuscript. We also thank L.A. Dew and L. Bocard for their contribution improving the language of the paper. This work was supported by fundings from ATM 'Formes possibles, formes réalisées' 2013 (SP, SL). We warmly thank Géraldine Veron and Céline Bens for access to the mammalian collection of the MNHN, Miguel Garcia-Sánz for technical support at the AST-RX platform, and Florent Goussard for his invaluable help in the Atelier 3D of the CR2P.

REFERENCES

Abràmoff MD, Magalhães PJ, Ram SJ. 2004. Image processing with ImageJ. *Biophotonics International* **11:** 36–42.

Abramov AV, Puzachenko AY. 2005. Sexual dimorphism of craniological characters in Eurasian badgers, *Meles spp.* (Carnivora, Mustelidae). *Zoologischer Anzeiger – A Journal of Comparative Zoology* **244:** 11–29.

Abramov AV, Puzachenko AY. 2006. Geographical variability of skull and taxonomy of Eurasian badgers (Mustelidae, Meles). *Zoologicheskii Zhurnal* **85:** 641–655.

Abramov AV, Puzachenko AY. 2013. The taxonomic status of badgers (Mammalia, Mustelidae) from Southwest Asia based on cranial morphometrics, with the redescription of *Meles canascens*. *Zootaxa* **3681:** 44–58.

Abramov AV, Puzachenko AY, Wiig Ø. 2009. Cranial variation in the European badger *Meles meles* (Carnivora, Mustelidae) in Scandinavia. *Zoological Journal of the Linnean Society* **157:** 433–450.

Adams DC, Rohlf FJ, Slice DE. 2004. Geometric morphometrics: ten years of progress following the 'revolution'. *Italian Journal of Zoology* **71:** 5–16.

Argyle EC, Mason MJ. 2008. Middle ear structures of *Octodon degus* (Rodentia: Octodontidae), in comparison with those of subterranean caviomorphs. *Journal of Mammalogy* **89:** 1447–1455.

Begg KS, Begg CM, Abramov AV. 2008. *Mellivora capensis. The IUCN Red List of Threatened Species 2008*. Available at: 10.2305/IUCN.UK.2008.RLTS.T41629A10522766.en

Billet G, Hautier L, Asher RJ, Schwarz C, Crumpton N, Martin T, Ruf I. 2012. High morphological variation of vestibular system accompanies slow and infrequent locomotion in three-toed sloths. *Proceedings of the Royal Society of London B: Biological Sciences*. **279:** 3932–3939.

Cattell RB. 1966. The scree test for the number of factors. *Multivariate Behavioral Research* **1:** 245–276.

Coleman MN, Colbert MW. 2010. Correlations between auditory structures and hearing sensitivity in non-human primates. *Journal of Morphology* **271:** 511–532.

Crumpton N, Kardjilov N, Asher RJ. 2015. Convergence vs. specialization in the ear region of moles (Mammalia). *Journal of Morphology* **276:** 900–914.

Cuvier G. 1825. *Discours sur les révolutions de la surface du globe. Troisième édition*. Paris: Dufour et d'Ocagne.

Davis H, Derbyshire AJ, Lurie MH, Saul LJ. 1934. The electric response of the cochlea. *American Journal of Physiology* **107:** 311–332.

Davison ML. 1983. Introduction to multidimensional scaling and its applications. *Applied Psychological Measurement* **7:** 373–379.

Elmeros M, Madsen AB, Prang A. 2005. Home range of the badger (*Meles meles*) in a heterogeneous landscape in Denmark. *Lutra* **48:** 35.

Fabre AC, Cornette R, Goswami A, Peigné S. 2015. Do constraints associated with the locomotor habitat drive the evolution of forelimb shape? A case study in musteloid carnivorans. *Journal of Anatomy* **226:** 596–610.

Fay RR. 1994. Comparative auditory research. In: Fay RR, Popper AN, eds. *Comparative hearing: mammals*. New York: Springer-Verlag, 1–17.

Fleischer G. 1978. Evolutionary principles of the mammalian middle ear. *Advances in Anatomy, Embryology and Cell Biology* **55:** 1–70.

Gittleman JL, Harvey PH. 1982. Carnivore home-range size, metabolic needs and ecology. *Behavioral Ecology and Sociobiology* **10:** 57–63.

Gridi-Papp M, Narins PM. 2009. Environmental influences in the evolution of tetrapod hearing sensitivity and middle ear tuning. *Integrative and Comparative Biology* **49:** 702–716.

Huang GT, Rosowski JJ, Flandermeyer DT, Peake WT. 1996. Middle ear of a lion: comparison of structure and function to domestic cat. *The Journal of the Acoustical Society of America* **99:** 2562–2574.

Huang GT, Rosowski JJ, Peake WT. 2000. Relating middle-ear acoustic performance to body size in the cat family: measurements and models. *Journal of Comparative Physiology A* **186:** 447–465.

Huang G, Rosowski J, Ravicz M, Peake W. 2002. Mammalian ear specializations in arid habitats: structural and functional evidence from sand cat (*Felis margarita*). *Journal of Comparative Physiology A* **188:** 663–681.

James FC, McCulloch CE. 1990. Multivariate analysis in ecology and systematics: panacea or Pandora's box? *Annual Review of Ecology and Systematics* **21:** 129–166.

Kruskal JB. 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika* **29:** 1–27.

Lay DM. 1972. The anatomy, physiology, functional significance and evolution of specialized hearing organs of gerbilline rodents. *Journal of Morphology* **138:** 41–120.

Legouix JP, Petter F, Wisner A. 1954. Étude de l'audition chez des mammifères à bulles tympaniques hypertrophiées. *Mammalia* **18:** 262–271.

Manley GA, Popper AN, Fay RR, eds. 2004. *Evolution of the vertebrate auditory system*. New York: Springer Handbook of Auditory Research 22, Springer-Verlag.

Mason MJ. 2004. The middle ear apparatus of the tuco-tuco *Ctenomys sociabilis* (Rodentia, Ctenomyidae). *Journal of Mammalogy* **85:** 797–805.

Nummela S. 1995. Scaling of the mammalian middle ear. *Hearing Research* **85:** 18–30.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H. 2014. vegan: Community ecology package. R package version 2.2-0. Available at: www.vegan.r-forge.r-project.org

Petter F. 1953. Remarques sur la signification des bulles tympaniques chez les Mammiferes. *Comptes rendus hebdomadaires des séances de l'Académie des Sciences* **237:** 848–849.

Pfaff C, Martin T, Ruf I. 2015. 'Septal compass' and 'septal formula': a new method for phylogenetic investigations of the middle ear region in the squirrel-related clade (Rodentia: Mammalia). *Organisms Diversity & Evolution* **15:** 721–730.

R Core Team. 2014. *R: a language and environment for statistical computing*. Vienna, Austria. Available at: https://www.r-project.org

Rabinowitz AR, Walker SR. 1991. The carnivore community in a dry tropical forest mosaic in Huai Kha Khaeng Wildlife Sanctuary, Thailand. *Journal of Tropical Ecology* **7:** 37–47.

Rose J, Moore A, Russell A, Butcher M. 2014. Functional osteology of the forelimb digging apparatus of badgers. *Journal of Mammalogy* **95:** 543–558.

Rosowski JJ. 1994. Outer and middle ears. In: Fay RR, Popper AN, eds. *Comparative hearing: mammals*. New York: Springer-Verlag, 172–247.

Salih WH, Buytaert JA, Aerts JR, Vanderniepen P, Dierick M, Dirckx JJ. 2012. Open access high-resolution 3D morphology models of cat, gerbil, rabbit, rat and human ossicular chains. *Hearing Research* **284:** 1–5.

Schleich CE, Busch C. 2004. Functional morphology of the middle ear of *Ctenomys talarum* (Rodentia: Octodontidae). *Journal of Mammalogy* **85:** 290–295.

Schleich CE, Vassallo AI. 2003. Bullar volume in subterranean and surface-dwelling caviomorph rodents. *Journal of Mammalogy* **84:** 185–189.

Shepard RN. 1962. The analysis of proximities: multidimensional scaling with an unknown distance function. I. *Psychometrika* **27:** 125–140.

Squarcia SM, Sidorkewicj NS, Casanave EB. 2007. The hypertrophy of the tympanic bulla in three species of dasypodids (Mammalia, Xenarthra) from Argentina. *International Journal of Morphology* **25:** 597–602.

Takechi M, Kuratani S. 2010. History of studies on mammalian middle ear evolution: a comparative morphological and developmental biology perspective. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* **314:** 417–433.

Terraube J, Arroyo B, Madders M, Mougeot F. 2011. Diet specialisation and foraging efficiency under fluctuating vole abundance: a comparison between generalist and specialist avian predators. *Oikos* **120:** 234–244.

Webster DB. 1962. A function of the enlarged middle-ear cavities of the kangaroo rat, *Dipodomys*. *Physiological Zoology* **35:** 248–255.

Webster DB. 1966. Ear structure and function in modern mammals. *American Zoologist* **6:** 451–466.

Webster DB, Webster M. 1980. Morphological adaptations of the ear in the rodent family Heteromyidae. *American Zoologist* **20:** 247–254.

Webster DB, Ackermann RF, Longa GC. 1968. Central auditory system of the kangaroo rat, *Dipodomys merriami*. *Journal of Comparative Neurology* **133:** 477–493.

Wever EG. 1974. The evolution of vertebrate hearing. In: Keidel WD, Neff WD, eds. *Handbook of sensory physiology. Auditory system*. Berlin, Heidelberg: Springer, 423–454.

Wible JR, Spaulding M. 2012. A reexamination of the *Carnivora malleus* (Mammalia, Placentalia). *PLoS ONE* **7:** e50485.

Zavattari E. 1938. Essai d'une interpretation physiologique de l'hypertrophie des bulles tympaniques des mammifères sahariens. *Mammalia* **4:** 173–176.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Scan parameters of each specimen: isotropic voxel size (μm, micrometres), voltage (kV), and current (μA, microamperes).

Table S2. Post-processing parameters of the image stacks for the two type of segmentations we made (entire skull, and bullae region): isotropic voxel size (μm, micrometres) and dimensions of the image stack.