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How Can Phenotypic Evolution be Characterized Over Time and Through Environmental Changes?

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Abstract

Rapid phenotypic evolution is observed in response to rapid environmental changes. These phenotypic variations can occur at different scales, from the population to the community. We intended to characterize these multiscale phenotypic responses in rodents from the archaeological site El Harhoura 2 (Rabat, Morocco), dated from the Late Pleistocene to the Holocene, and relate them to paleoenvironmental variations. Upper and lower first molars were used as proxies for phenotype. Their shape was quantified using a landmark-free method. To account for both intra- and interspecific variations, we used morphogroups as phenotypic units. Those morpho-groups were identified using unsupervised clustering. Three shape indicators were computed: number of morpho-groups, morpho-group disparity and changes in morpho-group mean shape over time. To evaluate this little-used approach on small mammals, we compared it to three widely used biodiversity indices: number of taxa, Shannon index, and Simpson index. Phenotypic evolution between and within species was highlighted. Morpho-groups seemed to be phenotypic response units representing ecological groups that transversed species. Variations in the morpho-group mean shapes were partly related to paleoenvironmental changes; however, variations in disparity were not. Thus, environmental changes deduced from fossil microvertebrate communities did not seem to be the main determinants of the characterized phenotypic variations.

Keywords: Phenotypic evolution · Paleoenvironments · Rodents · Teeth · Geometric morphometrics

Introduction

Climate change affects the living environments of organisms (vegetation, landscapes, etc.). These changes can be gradual or abrupt, and morphological variations at adaptative and/or phenotypical scales are often observed in response to these new environmental conditions (Lande 2009; Hoffmann and Sgro 2011; Clavel and Morlon 2017). Rapid phenotypic evolution in response to changes in the environment is related to microevolution and phenotypic plasticity (Holt 1990; Lande 2009; Hoffmann and Sgro 2011; Boutin and Lane 2014; Merila and Hendry 2014), two processes that generate interindividual variations (e.g., de Jong 1995; Fusco 2001). However, the phenotype also results from macroevolution that generates variations at the interspecific level (Erwin 2000; Hautmann 2020). Phenotypic evolution can thus refer differentially to intra- or interspecific evolution. The difference between those levels of variation is often not obvious because evolution is a continuous process, especially if there is an ongoing trend of divergence. Macroevolutionary changes are guided by microevolutionary changes occurring at each generation (Hansen and Martins 1996), and the uncoupling between these two processes is still being debated (Simpson 1944; Erwin 2000; Arnold et al. 2001; Hendry and Kinnison 2001; Reznick and Ricklefs 2009). To characterize the

phenotypic response to environmental changes, we chose to address phenotypic evolution as a composite process implying both intra- and interspecific evolution.

Most studies raising the issue of phenotypic evolution have used species as the phenotypic units to characterize phenotypic variation (e.g., Hendry et al. 2008; Bickford et al. 2010; Gardner et al. 2011; Reed et al. 2011; Boutin and Lane 2014; Radchuk et al. 2019). However, individuals belonging to the same species may display distinct phenotypic responses to environmental conditions, especially in a complex climatic context (Sultan and Spencer 2002). The concept of species itself, albeit discussed, defined as a morphologically or genotypically similar grouping of individuals, implies the existence of individuals with intermediate morphologies (Mayr 1942; Simpson 1951; Mallet 1995; Wheeler and Meier 2000; Baker and Bradley 2006). These intermediate morphotypes can display a variety of responses to environmental changes. When the addressed problematic goals are to identify patterns and/or characterize processes at a scale other than the species scale (as an intraspecific scale), this partition may not be appropriate. Therefore, in our case, that kind of approach is not the most suitable way to demonstrate phenotypic evolution on multiple scales. To detect phenotypic responses to environmental changes, we need to adopt a taxonomy-free approach.

In the context of our study, morphology is a potential candidate criterion to define those taxonomy-free units. Morphology as a phenotypic unit is particularly used in the field of study of foraminifera (e.g., Nagy 1992; Tyszka 1994; Alperin et al. 2011; Alegret et al. 2012; Khare et al. 2017). This shape-based approach is based on setting morphological groups (MGs) rather than taxa as biodiversity units. This approach groups individuals solely on the basis of their morphology. In the context of foraminifera, Murray (1973, 2006) defined MGs as phylogenetically independent groups of forms with similar test morphology. Since a test's form is closely related to its environment, this MG approach allows one to infer climatic conditions (Alperin et al. 2011). Conversely, it can be a relevant way to characterize phenotypic responses to environmental changes when studied organisms are known to be good environmental indicators. The concept of MGs has been successfully extended to other study frameworks, such as soil biocrust in Read et al. (2014), where they concluded MGs were functional response groups to environmental disturbances. Moreover, the MG approach presents demonstrated advantages: 1) it allows rapid and ecologically informative surveys of large morphological datasets; 2) it enables comparisons of assemblages of different ages; 3) taxonomic identification is not needed; and 4) MGs are independent of systematic relationships (Murray 1973, 2006; Nagy 1992; Read et al. 2014). We extended Murray's definition and defined MGs as phylogenetic independent groupings of forms with similar morphology.

El Harhoura 2 (EH2), a coastal cave located in the Rabat-Temara region. This site is of particular interest for the study of short-term phenotypic evolution related to environmental variations (Stoetzel 2009, 2017; Stoetzel et al. 2010, 2011, 2012b, 2017). This region is subjected to complex climatic influences from the Atlantic Ocean to the west, the arid Sahara to the south, and the Mediterranean region to the north. EH2 covers a time period from the Late Pleistocene to the Holocene, during which important climatic fluctuations occurred in the area (e.g., Drake et al. 2011, 2013; Blome et al. 2012; Kageyama et al. 2013; Scerri 2017; Couvreur et al. 2020). This variable climatic context resulted in a succession of relatively humid/arid and open/closed environments at EH2 (Stoetzel 2009; Stoetzel et al. 2011, 2012a, b). This cave revealed an exceptional richness in small terrestrial vertebrate remains accumulated through owl pellets and/or carnivore faeces, which have been intensively studied over the past decade (e.g., Michel et al. 2009; Stoetzel 2009; Stoetzel et al. 2010, 2011, 2012b, 2017; Cornette et al. 2015).

Among the small mammals of EH2, rodents are well represented in every layer. They are the most diverse group of living mammals (Carleton and Musser 2005) and are known for being good paleoenvironmental indicators (e.g., Avery 1982, Fernandez-Jalvo et al. 1998, McGuire 2010, Belmaker and Hovers 2011, Verde Arregoitia et al. 2017, Royer et al. 2020, Lopez-Garcia et al. 2021). Rodent morphology can thus reflect ecological similarities. Among most mammals, teeth display higher homoplasy than osteological parts (Evans et al., 2007; Brocklehurst and Benevento, 2020). Thus, rodent teeth may be a good proxy for estimating rodent phenotype. The shape-based grouping

of fossil rodent incisors has been shown to reflect dietary ecology (Paine et al. 2019), and molar crowns are strongly related to the environment through diet (Wolf et al. 2009; McGuire 2010; Coillot et al. 2013; Gomez Cano et al. 2013; Gomes Rodrigues 2015; Selig et al. 2021).

We studied the phenotypic evolution of the rodents of the EH2 cave using an MG approach to answer the following question: does short-term phenotypic evolution occur at different scales, i.e., intra/interspecific, under changing environments? We used two complementary models: first lower molars (m1) and first upper molars (M1). In murine rodents, both display slightly different evolutionary rates of size and shape (Renaud and van Dam 2002). MGs were identified using a non-subjective grouping method: unsupervised clustering (Hastie et al. 2009). For each dataset, three shape indicators were computed to evaluate phenotypic diversity: number of MGs, disparity over time and changes in mean shape over time. We expected MGs to not reflect species but to be representative of response groups towards environmental changes. We also expected to demonstrate both variations shared by several MGs under strong environmental changes (related to interspecific phenotypic evolution) and MG-specific variations when MGs display different phenotypic responses (related to intraspecific phenotypic evolution). The number of MGs and disparity should detect periods of decreasing/increasing/stable phenotypic diversity. Variations in mean shape should indicate shifts in selected shapes and the eventual convergence/divergence of MG shapes in constraining environments. However, the MG approach often underestimates diversity and can be costly in terms of information (Read et al. 2014). Then, to assess confidence in our approach, we compared its ability to characterize phenotypic evolution to widely used biodiversity indices in ecology and archaeology (number of species, Shannon index and Simpson index) (e.g., Avery 1982; Geraads et al. 2013; Meunier et al. 2020).

Material and Methods

El Harhoura 2 Cave

The stratigraphy of the cave is structured into 11 layers numbered from top to bottom (Fig. 1). Eight levels (L1 to L8) are well studied from their microfauna (Stoetzel et al. 2011, 2013, 2017) and well dated (Jacobs et al. 2012; Ben Arous et al. 2020a, b; Marquer et al. 2022) and were used in this study. A small mammal taxonomic study of the site reveals the presence of at least eight rodent species: *Apodemus sylvaticus*, *Lemniscomys barbarus*, *Mus spretus*, *Dipodillus campestris*, *Meriones shawii*, *Meriones grandis*, *Jaculus cf. orientalis* and *Eliomys cf. munbyanus* (Stoetzel et al. 2011, 2013, 2017). We were not able to include *Jaculus cf. orientalis* and *Eliomys cf. munbyanus* due to the lack of well-preserved teeth remains. Local paleoenvironmental information from EH2 is based on large and small vertebrates recovered from archaeological levels (El Hajraoui et al. 2012; Stoetzel et al. 2010, 2011, 2012b, 2014; Campmas et al. 2015; Stoetzel 2017). They show successive relatively humid (L3, L4a, L6 and L8) and arid (L2, L5 and L7) phases. Late Pleistocene environments appeared more open and less humid than those today. Paleolandscapes are defined as open steppe or savanna-like land with patches of shrubland, woodland and water bodies. Water bodies cover greater areas during humid phases than during arid phases (Stoetzel 2009).

Data Collection

We used 1133 m1s and 990 M1s housed at the Musée de l'Homme, Paris, France and the Institut National des Sciences de l'Archéologie et du Patrimoine, Rabat, Morocco. The sample was composed of six of the eight species present at EH2: *Meriones shawii*, *Meriones grandis* (referred to in the following as the *Meriones shawii/grandis* complex because of their morphological proximity (Stoetzel et al. 2017)), *Mus spretus* and *Dipodillus campestris* in abundance and occasionally *Lemniscomys barbarus* and *Apodemus sylvaticus*. The abundance of teeth per layer is indicated in Table 1.

Tooth photographs were taken using a Nikon digital camera D 5500 coupled with AF-S Micro NIKKOR 60 mm and macro extension tubes. Picture taking was standardized: teeth were always

placed so that the occlusal surface was horizontal. We obtained two categories of pictures: in situ teeth (when teeth were set in the mandible or the maxilla) and individual teeth (when teeth were no longer encased in the bone). In the first case, masks of teeth were manually extracted using the software Gimp v 2.10.6 (The GIMP Development Team 2018). In the second case, mask extraction was semi-automatized using ImageJ 1.52j software (Schneider et al. 2012) through the use of the segmentation procedure of the MorphoLibJ plugin (Legland et al. 2016), which automatically detects objects on a picture. The difference between those two acquisition methods was tested and was not statistically significant (Online Resource 1). The data acquisition protocol is summarized in Fig. 2. Finally, we extracted outlines from the masks using the library *Momocs* (Bonhomme et al. 2014) in the free software R (R Development Core Team 2018).

Geometric Morphometric Analyses

The tooth shape was approximated through 2D outlines of the teeth in occlusal view. Outlines were taken at the base of the molar crown, which is less affected by age. This is especially the case for *Meriones*, which has emihypsodont characteristics. As a consequence, the occlusal surface and pattern vary importantly with the age of the individual (as discussed in Stoetzel et al. 2017). Shape analyses were performed using the free software R (R Development Core Team 2018). Fourier analysis is an efficient method used to describe teeth morphology (Renaud 1999).

The registration process proposed in this article relies on an algorithm of functional generalized Procrustes analysis (FGPA), which is an extension of the GPA algorithm that can be found in Dryden and Mardia (1998: p. 90) but is adapted in cases where the outline of an object is considered a continuous closed curve. The idea behind this modification is to construct a landmark-free registration method avoiding the delicate choice of the number and the position of landmarks. Originally, tooth outlines were described by a different number of points depending on the specimen (between 1500 and 2000). Then, the contour detection algorithm performed an optimized resampling of 250 points. The final number of points was arbitrarily chosen to be the most informative while limiting the dimensionality of the data. Consider an observed closed contour of a tooth arriving as M pairwise coordinates $(x_1, y_1), \dots, (x_M, y_M)$. We first considered that a contour line could be expressed as two curves $(X(t), Y(t))$ that are supposed to be a linear combination of K known basis functions such that:

$$X(t) = \alpha_1 \phi_1(t) + \dots + \alpha_K \phi_K(t)$$

$$Y(t) = \beta_1 \phi_1(t) + \dots + \beta_K \phi_K(t),$$

where α_k s and β_k s are coefficients that must be estimated with regression on the sampled data, and K is the number of coefficients. The time index t arbitrarily belongs to $[0; 2\pi]$.

The basis functions $\phi_k, k = 1, \dots, K$ are chosen by the practitioner and constitute a Fourier basis in our case. Other choices (B-splines, polynomials, ...) can be relevant as well. The regression procedure was the same as that used in classical regression but in the functional case (see Ramsay and Silverman (2005) for more details) moving from sampled contours to continuous curves. The number K of basis coefficients was arbitrarily fixed in such a way that a sufficient amount of curve variability was captured. In our case, 30 basis coefficients (harmonics) were sufficient to describe the contour of the teeth, and additional coefficients did not provide more information.

Once every contour line was expressed through its estimated Fourier coefficients, it was possible to apply the registration FGPA algorithm using these coefficients as inputs. Registration of closed curves involves translating, rescaling, rotating and changing the phase of the configurations relative to each other to minimize an objective function (a total sum of squares). The registration of the starting points (phase changing) between curves was the main difference compared to the original Procrustes algorithm. They were not arbitrarily set and were part of the optimization process when registering the curves with Procrustes analysis, as shown in Fig. 3. See Online Resource 2 for more details about the registration algorithm.

Once the contour lines were registered, classical shape analyses could be achieved on the registered Fourier coefficients. To account for the entire shape variability, we used all Fourier coefficients in the following statistical analyses.

All subsequent treatments were applied to the two datasets (m1 and M1) separately, and all species were analysed together using the same protocol. To reduce data dimensionality in further analyses, we performed principal component analysis on both datasets using the *PCA* function of the *FactoMineR* package (Le et al. 2008). We retained 95% of the shape variability, and the latest principal components are usually considered neglectable (Baylac and Frie. 2005). In total, we used 20 principal components for the m1 dataset and 20 for the M1 dataset. As an estimate of tooth size, we used the surface area of masks.

Allometry

Allometry is the part of the shape caused by the influence of size (Gould 1966; Klingenberg 1996, 2016). Size variation is an important determinant of morphological changes and consequently plays a critical role in the evolution of shape (Klingenberg 2016). If the FGPA procedure separates shape from size, it does not remove allometry. The presence of allometry was tested by performing the multivariate regression of shape on size using the function *procD.lm* from the library *geomorph* (Adams and Otarola-Castillo 2013).

Clustering Analyses

To partition datasets without any prior information, we performed unsupervised clustering analyses based on shape variables. We chose to explore two complementary clustering protocols (summarized in Fig. 4):

1. Method 1 partitioned the global variability into MGs and gave an overview of MG evolution over time by retaining identity between MGs from one layer to another. However, this method implies that the phenotypic evolution that occurred in MGs over the time interval considered is less important than the phenotypic difference between MGs. Clustering was applied to the overall datasets.

2. Method 2 partitioned variability on each layer separately and thus considered phenotypic evolution occurring over the considered time interval. In contrast to method 1, method 2 is not based on any assumption about phenotypic evolution in MGs. However, it does not allow us to follow MG evolution over time because the relationship of MG identity determined in different layers is not known. The clustering was applied independently on each layer.

Clustering analyses were performed using the unsupervised morphological K-nearest neighbours method (KNN). KNN is a nonparametric classification (i.e., a method that is not based on statistical distributions) and therefore was applicable to both of our approaches (each of them implying highly varied sample sizes). The KNN classification is based on the assumption: "tell me who your neighbours are, and I will tell you who you are". In other words, each shape object is affected by its nearest neighbours' clusters. Unsupervised KNN was implemented in the *clues* function of the *clues* package (Wang et al. 2007). The dissimilarity measure used was Euclidean. In *clues*, the number of clusters and K (the number of neighbours to consider) were estimated by the algorithm. The number of clusters was obtained thanks to a partition procedure preceded by a local shrinking procedure, in which data points were "shrunk" towards a cluster centre (Wang et al. 2007). Then, K was selected between 1 and n-1 (n being the number of objects) based on the more robust clustering result (Wang et al. 2007). This robustness was assessed by the Silhouette index (SI) (Kaufman and Rousseeuw 1990), which measured the strengths of clusters. The SI was determined for each data point. It is comprised between -1 and 1. If $SI > 0$, the data point is closer to its assigned cluster than to other clusters. If $SI < 0$, the data point is misassigned to its cluster. If $SI = 0$, the data point is at equal distance from its assigned cluster and neighbouring clusters. To ensure that points are correctly allocated to clusters, the average SI was calculated and must be strictly positive (Wang et al. 2007).

Clustering results and species are presented on PCA per layer in Online Resources 3, 4, 5 and 6. Size differences between MGs were tested using a one-factor ANOVA (analysis of variance) performed with the *aov* function of the *stats* library.

Shape Indicators

We studied three shape indicators over time: number of MGs, disparity and mean shape.

1. The number of MGs is the number of significantly distinct morphological units in a dataset. This value was obtained directly from the clustering analysis.

2. Disparity is the range of morphological variety in a group. It indicates changes in the amount of variation of a morphotype (Gould 1989, 1991; Wills et al. 1994; Foote 1997). When considered independently of phylogenetic relationships, a good estimate of disparity is to quantify the amount of occupied morphospace (Wills et al. 1994). As a disparity estimator, we used the median of the distances from the centroid of the group, a variance measure relatively insensitive to outliers (Guillerme et al. 2020). Disparity was obtained using the function *dispRity.per.group* from the package *DispRity* (Guillerme 2018). This function contains a bootstrap procedure to reduce the effect of sample composition, a common issue when studying disparity (Butler et al. 2012). Regarding sample size, variance is generally not biased by it (Foote 1997), and no sample size correction was applied. We tested the statistical significance of disparity variation between the types of environments and between EH2 layers. Regarding method 1, we performed Friedman tests. This test is a rank sum test often used as an alternative to one-factor ANOVA on paired samples when the normality assumptions are not met. We used the *friedman.test* function from the *stats* library. Regarding method 2, we performed Kruskal–Wallis tests, a test similar to the Friedman test but adapted to independent samples. We used the *Kruskal.test* function from the *stats* library.

3. Mean shape reflects changes in the global shape of a group over time. It can detect shape divergence in groups in some layers. To quantify shape variations, we built a distance tree between mean shapes per MG per layer. We computed Mahalanobis distances and built the tree using the neighbour-joining algorithm, an agglomerative clustering method. The distance between groups in the tree was proportional to the morphological differences. To do that, we used the functions *dist* from the package *stats* and *nj* from the package *ape* (Paradis et al. 2004). Visualizations of mean shapes per MG were also obtained. The neighbour joining distance trees were computed only for MGs obtained with method 1, as the MGs of method 2 had no continuity between layers. For each MG, we also tested the statistical significance of mean shape variation between the types of environments and between EH2 layers. We performed MANOVA (multivariate analysis of variance) and conducted pairwise Hotelling's T2 tests (the multivariate alternative to the t test). To correct the Hotelling's T2 results for multiple testing, we applied Bonferroni's criteria, which consists of identifying the number of tests n and then performing each of the n tests not at the α level of significance but at the α/n level. We used the *manova* function of the *stats* library and *hotelling.test* function from the *Hotelling* library (Curran 2017).

Biodiversity Indices

Biodiversity indices are frequently used in ecology, and some of them, such as the Shannon and Simpson indices, are also often applied to rodent fossil assemblages (Avery 1982; Geraads et al. 2013; Meunier et al. 2020). Those indices compute quantitative parameters about the structure of the specific diversity of a faunal assemblage (such as species richness, diversity and specific dominance). We choose three diversity indicators:

1. Number of taxa over time.

2. The Shannon–Weaver index (H'), which allows the estimation of the diversity of a community of organisms. A value close to 0 indicates that only one species is represented or that most organisms belong to the same species. The index is highest when many species are represented and when organisms are well distributed among species (Blondel et al. 1973; Hill 1973). This index is sensitive

to variations in abundance of rare species (Peet 1974) and highly sensitive to sample size when it is composed of fewer than 25 individuals (Cruz-Urbe 1988) (which was not the case here).

3. The Simpson index (D) measures the probability that two randomly selected individuals are of the same species. We used the unbiased Simpson index (D'), which is corrected for sample size, as suggested in Faith and Du (2018). It ranges from 0 (when diversity is maximized) to 1 (when diversity is minimized). The transformation $1-D'$ provides values that are easier to manipulate and interpret (Pielou 1969; Hill 1973). This index is sensitive to variations in abundance in more important species (Peet 1974). Biodiversity indices were computed for Rodentia alone and for all terrestrial microvertebrates, including Rodentia, Squamata, Amphibia, Chiroptera, Soricomorpha, and Erinaceomorpha, based on data from Stoetzel (2009) and Stoetzel et al. (2011, 2012b).

Results

Allometry

A significant, albeit weak, allometric signal was found in both rodent m1 ($R^2 = 0.09$; p value = 0.001^{**}) and rodent M1 ($R^2 = 0.10$; p value = 0.001^{**}).

Clustering Analyses

The clustering results for both methods and both datasets are summarized in Table 2. All values of the SI means were in the range of (0,1), meaning data points were rightly assigned to their clusters. The detailed composition of each MG is available in Online Resources 7, 8, 9 and 10.

With method 1, we identified three MGs in the m1 dataset. MG1 contained mostly *Mus spretus* teeth but also some *Dipodillus campestris*, *Meriones shawii/grandis* and *Apodemus sylvaticus* teeth. MG2 and MG3 contained mostly *Meriones shawii/grandis* teeth but also some *Dipodillus campestris* and *Mus spretus* teeth. In the M1 dataset, two MGs were identified. MG1 was transgeneric and contained *Mus spretus* and *Meriones shawii/grandis* teeth, while MG2 was mainly composed of *Meriones shawii/grandis* teeth. MGs displayed significantly different sizes for m1 (ANOVA: p value < $2e-16^{***}$; $df = 2$; $F = 355$) and M1 (ANOVA: p value < $2e-16^{***}$; $df = 1$; $F = 70.57$).

For method 2, no identity between MGs from different layers was established. We identified up to three MGs per layer in the m1 dataset. Except for L7, all layers displayed only two MGs. Half of the MGs were transgeneric, while the other half were mainly composed of *Meriones shawii/grandis* teeth. In the M1 dataset, up to five MGs were identified. For m1, half of the MGs were transgeneric, while the other half were mainly composed of *Meriones shawii/grandis* teeth. MGs displayed significantly different sizes for m1 (ANOVA: p value < $2e-16^{***}$; $df = 1$; $F = 145.9$) and M1 (ANOVA: p value < $2e-16^{***}$; $df = 1$; $F = 95.82$).

MG size over time was explored and found to be invariant and thus uninformative with respect to our research question. Consequently, those results are neither presented nor discussed. For additional information, boxplots of lower and upper molar sizes per species are available in Online Resource 11.

Shape Indicators

The number of MGs and the disparity obtained for m1 over the eight layers of EH2 are presented in Fig. 5, and those obtained for M1 are presented in Fig. 6. Mean shape distance trees are presented in Fig. 7. For clarity, each analysis result is referred to as the "dataset-method", as in m1-method1.

The results showed several significant phenotypic variations.

Global disparity variation over EH2 layers was statistically significant for m1-method1 (Friedman test: p value = 0.017^* , $df = 7$, $X^2 = 17$) but not for M1-method1 (Friedman test: p value = 0.19 , $df = 7$, $X^2 = 10$), m1-method2 (Kruskal–Wallis test: p value = 0.21 , $df = 7$, $X^2 = 9.66$) or M1-method2 (Kruskal–Wallis test: p value = 0.78 , $df = 7$, $X^2 = 3.97$). In m1-method1, we observed a global increase

in disparity from L8 to L4a, followed by an important decline between L4a and L3 and then a slight increase until L1 was shared by all MGs (Fig. 5b). If we focused on MG disparity, this pattern of variation was also present in one/several MGs of m1-method2 (Fig. 5d) and in MG2 of M1-method1 (Fig. 6b). The variation in global disparity between environmental types was not statistically significant for m1-method1 (Friedman test: p value = 0.45, df = 3, $X^2 = 2.6$), M1-method1 (Friedman test: p value = 0.14, df = 3, $X^2 = 5.4$), m1-method2 (Kruskal–Wallis test: p value = 0.21, df = 3, $X^2 = 4.6$) or M1-method2 (Kruskal–Wallis test: p value = 0.98, df = 3, $X^2 = 0.19$).

Concerning the number of MGs over time, there was an increase in m1-method 2 and M1-method 2 in L7 (Figs. 5c, and 6c), the only layer characterized by an open and arid environment. In M1-method 2, there was also an additional MG in L5 (Fig. 6c), a layer characterized by an arid and semi-open environment.

Regarding mean shapes, the MGs of method 1 did not display the same internal shape variability. In m1-method1, MG1 showed more similar mean shapes, while MG3 had more diverse mean shapes (Fig. 7a). Similarly, in M1-method1, MG2 was composed of more similar mean shapes (except for the one in L1) than MG1 (Fig. 7b). In L3, the MGs of M1-method1 showed more similar mean shapes than those in other layers (Fig. 7b). Over the EH2 layers, the mean shape variation within each MG was statistically significant for m1-method1 (MANOVA: MG1: p value = $1.68e-9$ ***, df = 7, F = 1.91, Pillai = 0.53; MG2: p value = $1.94e-8$ ***, df = 7, F = 1.84, Pillai = 0.60; MG3: p value = $4.31e-06$ ***, df = 7, F = 1.67, Pillai = 0.95) and for M1-method1 (MANOVA: MG1: p value = $4.76e-8$ ***, df = 7, F = 1.81, Pillai = 0.68; MG2: p value < $2.2e-16$ ***, df = 7, F = 3.069, Pillai = 0.64). The results of pairwise Hotelling's T2 tests are available in Online Resource 12. Variations between environmental types were also statistically significant for m1-method1 (MANOVA: MG1: p value = $1.61e-09$ ***, df = 8, F = 2.56, Pillai = 0.30; MG2: p value = 0.00058 ***, df = 8, F = 1.73, Pillai = 0.24; MG3: p value = 0.00093 ***, df = 8, F = 1.72, Pillai = 0.42) and for M1-method1 (MANOVA: MG1: p value = 0.0023 **, df = 8, F = 1.63, Pillai = 0.26; MG2: p value < $2.2e-16$ ***, df = 8, F = 4.30, Pillai = 0.37). The mean shape differences between environments per MG showed that the different MGs were not sensitive to the same environmental differences (Fig. 7). The results of pairwise Hotelling's T2 tests are presented in Online Resource 13.

Biodiversity Indices

Biodiversity indices over the eight studied stratigraphic layers of EH2 are presented in Fig. 8. All indicators displayed a continuous increase in diversity from L8 until L5-L4a, then a decrease until L2 and finally a second increase in L1 (Fig. 8). This general trend was nuanced in L7 by a decrease in the number of taxa of terrestrial vertebrates (Fig. 8A) and in L6 by a decrease in rodent diversity indicated by the Shannon and Simpson indices (Fig. 8b and c).

Discussion

We aimed to assess short-term multiscale phenotypic evolution in EH2 cave rodents relative to environmental changes. To do so, we studied the shape variation of rodents' teeth over time through an approach that set MGs instead of taxa as the phenotypic units. First, we will discuss the evidenced phenotypic variation over time. Then, we will discuss our methodological approach.

Phenotypic Variations Under Changing Environments

The main disparity variation consisted of an increase from L8 to L4a followed by a very large diversity drop between L4a and L3 and then an increase until L1 (around the late Pleistocene-Holocene transition). This result is illustrated by the disparity variations of the three MGs of m1-method1 and of MG2 of M1-method1, meaning there were important changes in the amount of variation within concerned morphotypes. The Shannon index of terrestrial microvertebrates displayed similar variation over time. This result supports the idea that the morphological variability

in rodent teeth is consistent with variations in terrestrial microvertebrate diversity. Moreover, it demonstrated that the exclusion of some rare species from our sampling (*Jaculus cf. orientalis* and *Eliomys cf. munbianus*) did not significantly affect our results.

The ecomorphological meaning of those variations in disparity depends on the ecological and functional significance of the studied morphological features (Ricklefs and Miles 1994; Van Valkenburgh 1994). In the case of teeth, because morphological changes can be related to the environment through diet (Wolf et al. 2009; McGuire 2010; Coillot et al. 2013; Gomez Cano et al. 2013; Gomes Rodrigues 2015; Pineda-Munoz et al. 2017), changes in disparity/diversity patterns can reflect environmental variations (Erwin 2007). The fact that disparity variations in our results were displayed by two morphological features (m1 and M1) and were shared by MGs that included diverse species (*Meriones shawii/grandis*, *Dipodillus campestris* and *Mus spretus*) suggested that it was driven by (an) external disturbance(s). However, it is difficult to associate specific phenotypic responses to particular environments. Moreover, no major environmental change was identified by paleoenvironmental studies between L4a and L3, while an important variation in disparity was identified. Moreover, clades other than rodents were impacted: variations in shrew species composition have been observed in the same layers (Cornette et al. 2015). Thus, the considered environmental conditions do not seem to be entirely related to disparity variations. Either disturbances of another nature— ecological or anthropogenic—must have caused those phenotypic changes, or there have been environmental changes that could not be detected through the usual markers used in paleoenvironmental inferences. The disparity variation of m1-method1 may support this last hypothesis, as it was statistically significant between layers but not between environmental types.

This mismatch might be explained by the fact that paleoenvironmental inferences can sometimes be questionable. At EH2, paleoclimatic fluctuation assessments were based on standard analyses of microvertebrate assemblages and were complemented by stable carbon (¹³C) and oxygen (¹⁸O) isotope analyses on *Meriones shawii/grandis* teeth (Jeffrey 2016). These analyses indicated that L11 to L7 (dated to MIS 5) encountered more humid conditions than today, while L6 to L3 (dating from MIS 5 to 3) were subjected to conditions similar to the present conditions. This result suggests a relatively open environment throughout the Late Pleistocene. L2 (dated to MIS 2 and/or beginning of MIS 1) appeared more arid, which was consistent with the aridification of the region at this period. We thus observed some discrepancies with the previous palaeoecological data, notably in L7, L6 and L5. Isotopic results indicated relatively humid conditions in L7 and L5 and more arid conditions in L6, while faunal communities indicated the opposite. These discrepancies could be explained by different phenomena. One of these could be the differences in the signals recorded from a set of species communities and those recorded from individuals within a single species (which can adapt differently to environmental changes) (Stoetzel et al. 2019). Consequently, the paleoenvironmental signal at the individual or species/MG scales may be different than that at the community scale.

An alternative explanation for this mismatch might reside in the fact that dental morphology reflects the environment through diet (Wolf et al. 2009; McGuire 2010; Coillot et al. 2013; Gomez Cano et al. 2013; Gomes Rodrigues 2015; Selig et al. 2021). However, the dietary preferences of the species considered may not be directly linked to environmental inferences. Some rodent species in our sample have flexible diets (e.g., Palomo et al., 2009; Adamou-Djerbaoui et al., 2013) and thus can display broad dietary categories. Then, their diet composition depends not only on resources available but also on other ecological factors, such as competition [Ledevin et al. (2016) indicates that competition with other species may be a more important driver of insular mouse molar shape than the environment. Consequently, the shape indicators computed on MGs and environmental inferences may not be related to the exact same environmental and ecological parameters. Moreover, MGs may not be exclusively partitioned on ecological characteristics; rather, they may also be partitioned on life history traits, meaning they may not display equal phylogenetic signals. For example, MG2 of M1-method1 was mainly composed of one species complex, *Meriones shawii/grandis*, suggesting that it might have been clustered based on traits that reflect history. This would explain why MG was not correlated with environmental inferences.

Regarding the m1 dataset, the MG comprising *Mus spretus* (MG1) displayed significantly similar mean shapes per layer (except in L1). This result suggests a high morphological stability of this MG over time and with environmental changes. This result is consistent with the continuous presence of this species over the EH2 sequence, with no significant changes in terms of abundance over time (Stoetzel et al. 2011) and a stability in morphology and size of molars from the Late Pleistocene to the Middle Holocene (Stoetzel et al. 2013). This result may be related to a strong adaptability of this generalist species to environmental changes and/or a continuous presence of suitable habitats for this species in the Rabat-Temara region through the Late Quaternary (Lalis et al. 2019). Geometric morphometric analysis of *Meriones* molars has shown that during the Late Pleistocene, both *M. shawii* and *M. grandis* were present in western Morocco (Stoetzel et al. 2017). Few changes occurred throughout this period despite several climatic changes, indicating that suitable habitats probably always occurred in the region, as was the case for *Mus spretus*. However, during the Middle Holocene, paleontological and morphometric analyses highlighted a change in the faunal spectrum and species distribution accompanied by a clear demographic collapse (Lalis et al. 2016; Stoetzel et al. 2017). The authors therefore suggest that during the humid period of the Middle Holocene, when Mediterranean forests expanded, the semiarid open steppe habitats, which were more suitable for *Meriones*, decreased drastically in the northern half of Morocco, leading to population collapses and changes in their geographic distribution. Consequently, we hypothesize that *Mus spretus* may have been less affected by environmental changes than *Meriones shawii/grandis* and that the identified disturbance(s) in L4a must be of a climatic nature. This result is supported by isotope studies that display higher $\delta^{13}\text{C}$ values than the normal values observed at EH2, possibly indicating an important change in vegetation at this time (Jeffrey 2016; Stoetzel et al. 2019). Another possibility would be to relate this change in fauna with predator activity. The presence of variable nonhuman predators (especially small mammalian carnivores and birds of prey) has been attested at EH2 cave and is suspected to be the main agent of modification for faunal remains (Stoetzel 2009; Campmas et al. 2015, 2017). Even if the microvertebrate diversity does not appear to be related to predator shifts between levels (Stoetzel 2009; Stoetzel et al. 2011), microevolutionary trends might have been affected, thereby influencing macroevolutionary trends (Hansen and Martins 1996).

The combination of the different shape indicators can allow us to formulate hypotheses about evolution scenarios. This was the case for the phenotypic changes in M1-method1 that occurred in L3, and especially MG2. In this layer, there is a consistent decline in disparity, combined with a mean shape convergence between the two MGs. Mean shapes represent clumps of forms in the occupied morphospace and disparity the dispersal of those clumps. It is known that morphological clumps can represent local optima surrounded by less fit alternatives (Erwin 2007). Shifts in mean shapes should then happen when there is a shift in ecology and thus in optima. Moreover, changes in disparity patterns can reveal patterns of morphological selectivity (Erwin 2007). Thus, a reduction in disparity combined with a mean shape shift towards a particular morphotype can be interpreted as the selection of a form that is advantageous with respect to environmental conditions. Then, we can hypothesize that a morphological selective event occurred in L3, resulting in a shape convergence between the two MGs.

In L7 in m1-method 2 and M1-method 2, an increase in the number of MGs coupled with a decrease in the disparity of some MGs was observed. The presence of a new morphotype might have reinforced competition for resources, resulting in each morphotype converging towards its mean shape, reducing each group's disparity. Faunal variations in this layer were also observed in shrews with a particular species composition in the layer (Cornette et al. 2015). This result might be explained by a particularly arid and open environment at this time (Stoetzel 2009; El Hajraoui et al. 2012). However, isotope studies disagree with this hypothesis and suggest there were more humid conditions (Jeffrey 2016). The hypothesis of an arid climate in L7, as in L5, is mainly supported by the species abundance and the presence of *Jaculus cf. orientalis*. However, this steppic species can also be considered an indicator of more continental conditions (i.e., shoreline retreat caused by a sea-level drop) rather than of particularly arid conditions. Consequently, some arguments question the

paleoenvironmental inferences in L7 based on faunal communities, even if it is almost undeniable that an environmental change occurred.

In L6, MG1 of M1-method1 displayed a very large decline in disparity. An important fact to underline is that L7 and L6 (as for L4a and L3) are very close layers in the data (Jacobs et al. 2012; Janati-Idrissi et al. 2012; Ben Arous et al. 2020a, 2020b). Thus, these phenotypic variations have a unique cause. We must also keep in mind that two species, which we know are present in these deep layers, were not included in this study (*Jaculus* cf. *orientalis* in L5 and L7 and *Eliomys* cf. *munbyanus* in L5 and L8). Therefore, the importance of these trends might be underestimated by this bias in the sampling.

Lower and Upper Molars, Two Complementary Models

The shapes of the two biological objects—m1 and M1— globally reflected evolutionary patterns similar to those observed in other rodent species (e.g., Renaud et al. 1999). More diversity was identified among m1, but more varied trends were displayed by M1. Statistical tests of the mean shape differences between environmental types indeed showed that M1 was more sensitive to environmental changes than was m1. However, these results were eventually less informative because they were less specific to particular environmental transitions. Thus, we did not find a clear higher responsiveness of M1 to environmental changes, contrary to what was concluded by Renaud et al. (2011). However, this result makes sense, as Renaud et al. (2011) explained this stronger responsiveness was due to the fact that M1 has a higher allometric component than m1, inducing a greater evolvability of M1, and that in our dataset, both M1 and m1 display similarly weak allometry. Indeed, we found that tooth size explained only ~ 10% of shape variation in both m1 and M1, which was quite low but was within the range of allometry usually found in rodent teeth (e.g., ~ 3% in insular mice, as found by Ledevin et al. (2016), and ~ 20% in the Orkney vole, as found by Cucchi et al. (2014)). Each of the biological models allowed us to detect different particular trends: different phenotypic responses within one species in the case of m1 and, for M1, a morphological selective event in L3. These two phenotypic proxies thus provide complementary information leading to a better understanding of rodent phenotypic evolution.

Biological Interpretation of the MGs

The MGs are taxon-free groups of specimens with more similar teeth shapes and significantly different sizes. They were also characterized by phenotypic evolutionary characteristics. For example, the MGs of m1-method1 displayed varied mean shape similarities between layers. This could be interpreted as different degrees of morphological stability over time and through varied environments. MG1 had significantly similar mean shapes (except in L1), possibly implying a high resistance to external variations. Conversely, MG2 had more significantly different mean shapes between layers, meaning that this MG was more plastic. Moreover, MGs were not equally sensitive to all environmental changes, suggesting that they were responsive to different environmental parameters. MG1 of m1-method1 was sensitive only to transitions between humid and close environments to all other types of environments. MG1 of M1-method1 was very insensitive to changes in the environment, while MG2 was highly sensitive. Thus, MGs seemed to have different responses to disturbances. They might be interpreted as taxon-free ecological response groups, which can be expected because rodents are ecological opportunists with flexible diets and habitat preferences (Nowak 1999; Alhajeri and Stepan 2018).

We detected more phenotypic variations than what is evidenced by conventional indicators of biodiversity, although our results remain consistent with the latter. This may be explained by the fact that during the Late Quaternary, environmental changes were less abrupt in North Africa than in Europe, and no major faunal turnover was observed. The geographical location of the EH2 cave also implies that fauna and vegetation are strongly influenced by the Atlantic ocean, which was probably the case during the whole Quaternary. This includes fewer variations in humidity and temperature on

the coast than in the interior of the continent, as well as regional faunal differences (Stoetzel 2017; Stoetzel et al. 2019). In sum, only minor changes in microvertebrate communities were observed throughout the EH2 sequence (Stoetzel et al. 2011; Stoetzel 2013, 2017). This means that a 'common pool' of species was preserved through time with only a few differences in the composition and proportions of species. Microvertebrates experienced low-amplitude environmental changes, although alternations between arid and humid periods were recorded at EH2. The landscapes would have been characterized by a mosaic of habitats, with only the relative cover of the different habitats changing over time. Most of these changes have certainly been too subtle to be detected by the classic biodiversity indices.

In an archaeological context, this approach may represent another way of apprehending phenotypic diversity. It is recognized that in archaeology, global diversity is often underestimated because of the difficulty in identifying some taxa (Stoetzel 2009). Moreover, even current taxa taxonomy can be unstable, as for the *Meriones shawii/grandis* complex, the systematic nature of which has been very controversial for a long time (Carleton and Musser 2005; Darvish 2011; Lalis et al. 2016; Stoetzel et al. 2017). The MG approach is independent of identification, and in some cases, unsupervised clustering proved to better evaluate phenotypic diversity than species (Quenu et al. 2020). Moreover, the association of geometric morphometrics and machine learning has repeatedly proven its ability in terms of phenotype discrimination (Dubey et al. 2006; Bocxlaer and Schulthei. 2010; Cornette et al. 2015; Guillaud et al. 2016; Mapp et al. 2017; Soda et al. 2017; Fang et al. 2018; Quenu et al. 2020). As a consequence, it might be a complementary local diversity indicator to species, as it does not carry the same information and potentially represents ecological groups.

However, this approach has the disadvantage of being a relative rather than an absolute characterization of phenotypic diversity, which could sometimes lead to an underestimation of the overall diversity when compared with other localities (Read et al. 2014). Moreover, we must keep in mind that *Jaculus cf. orientalis* (present in L5 and L7) and *Eliomys cf. munbyanus* (present in L5 and L8) were not considered in the present study because of the lack of usable teeth for geometric morphometric analyses, which could have led to an underestimation of the diversity in the deepest levels.

The MG approach proposed in this paper has a second disadvantage: the *sine qua non* assumption of method 1. Herein, the MG approach was addressed through two complementary methods. The major difference between the two is that method 1 partitions the variability once on the overall dataset, while method 2 does it layer by layer. By pooling remains from different layers, the partitioning protocol of method 1 implies that phenotypic evolution occurring in MGs over time is less important than the phenotypic difference between MGs. It is questionable whether this hypothesis is fully satisfied. First, the number of MGs varied greatly between the two methods. Phenotypic evolution had already been brought to light over the studied time interval (i.e., between layers). Indeed, morphological differences were observed in remains of the same taxon between old and recent layers. These differences were attributed to the presence of "primitive" morphological characters in old layers (Stoetzel 2009). However, the overall similarity of trends between the two methods suggests that it is doubtful that those phenotypic changes are equally or more important than phenotypic differences between MGs. The case of EH2 rodents seems to meet the hypothesis of method 1 to some extent. However, this was not the case for any dataset, and the MG approach will be applicable only to a limited number of cases.

Conclusion

We observed significant short-term shape changes within MGs at both intra- and interspecific scales and identified significant variations in MG disparities over time. Thus, MGs appeared to be phenotypic response units that could be transversal between species. However, environmental changes did not seem to be the main determinants of characterized phenotypic variations, as they were not related to changes in disparity. Nevertheless, discrepancies between paleoenvironmental proxies might suggest the existence of some uncertainties in paleoenvironmental inferences that

might explain our results. Another lead may be to look at the functional characteristics of MGs rather than the morphological ones. Because they more directly represent the ability of individuals to perform ecologically relevant tasks, they might allow one to obtain a better characterization of the phenotypic responses to environmental changes.

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Availability of Data and Code. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. The R script used for analyses is available in Online Resource 14.

Declarations

Conflict of Interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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FIGURES

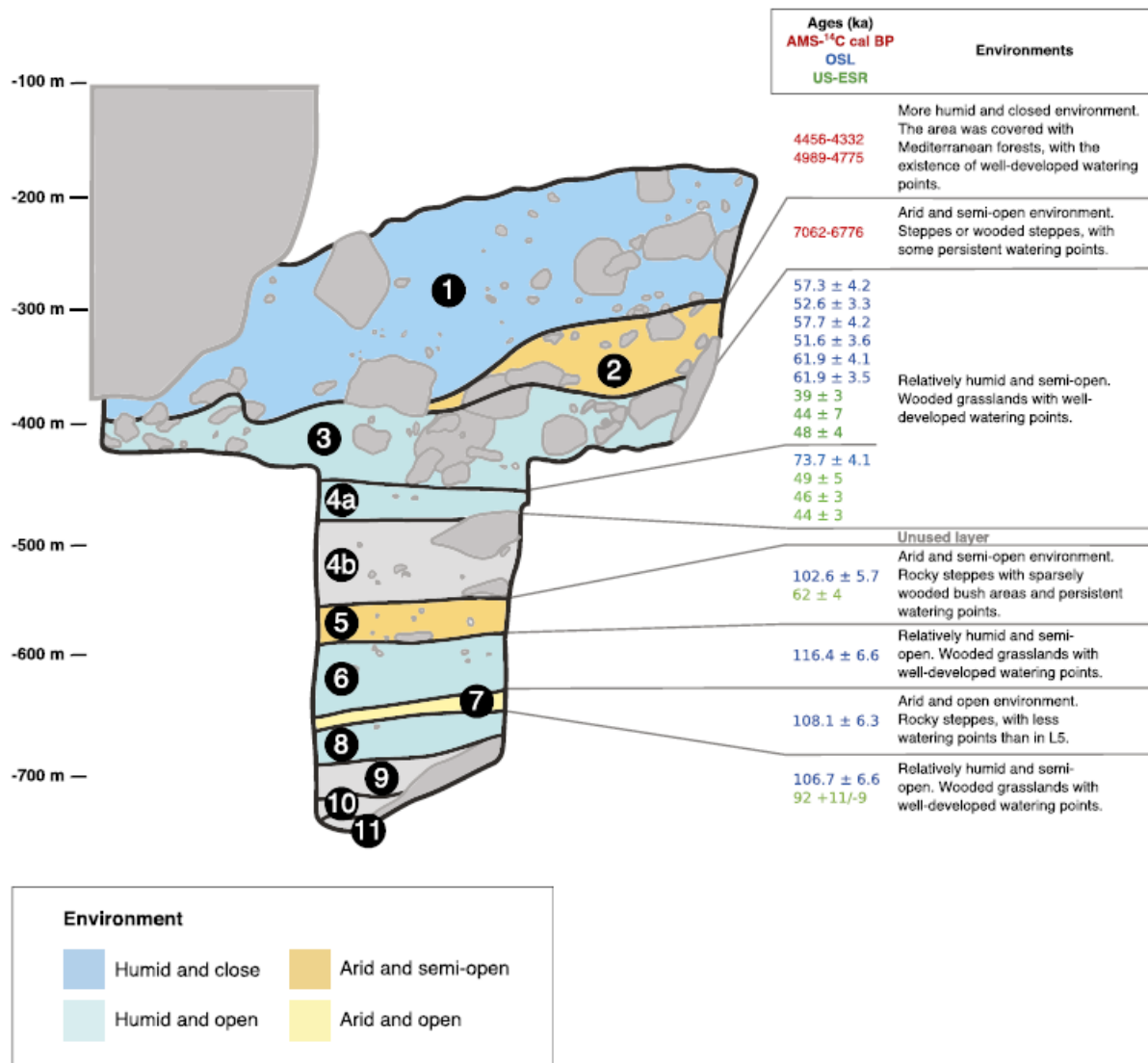


Fig. 1. Stratigraphy, age, and characteristic environments of the eight layers of EHZ2 used in this study, color-coded by environment (Michel et al. 2009; Stoetzel 2009; Jacobs et al. 2012; Janati-Idrissi et al. 2012; Nespoulet and El Hajraoui 2012; Ben Arous et al. 2020a, b; Marquer et al. 2022). Three dating methods were used: AMS-14C based on organic remains, combined US-ESR (Combined uranium series and electron spin resonance) and OSL (optical stimulated luminescence). Layers unused in this study are in grey.

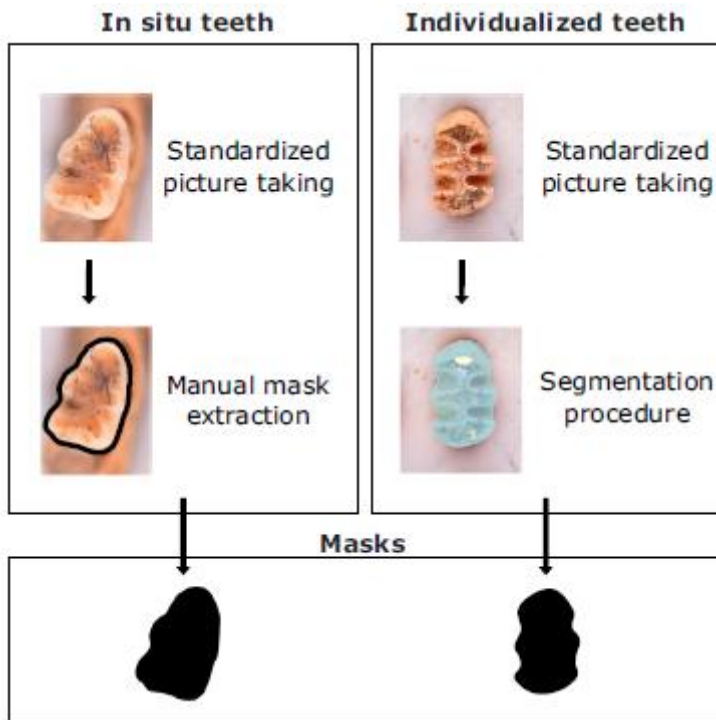


Fig. 2. Acquisition protocol from picture taking to mask extraction. The protocol varied depending on whether the tooth was individualized or not (i.e., separate or still set into the mandible or the maxilla).

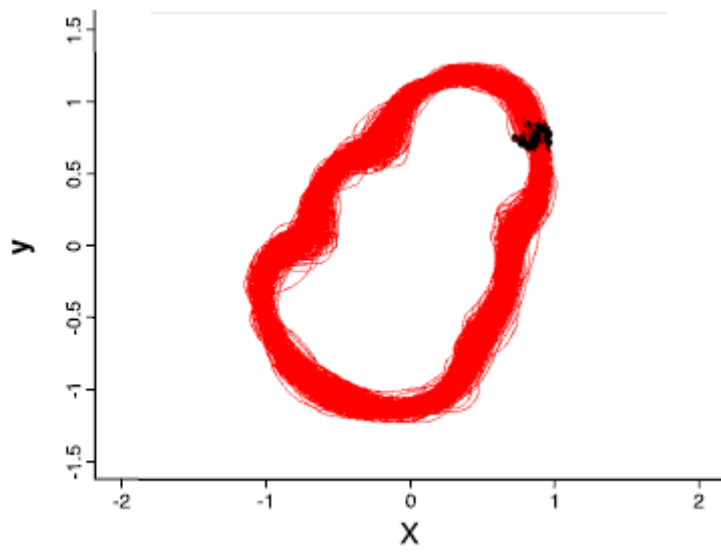


Fig. 3. Aligned outlines of right lower first molars with the registered starting point of each curve.

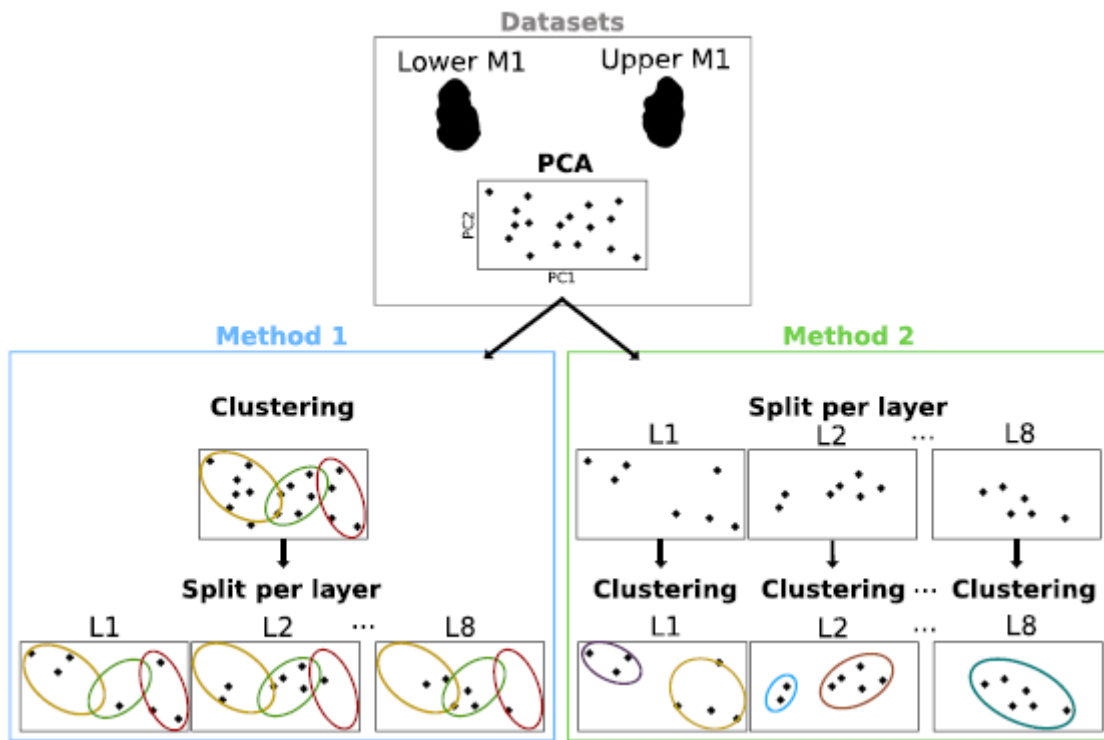


Fig. 4. Protocols of the two clustering methods.

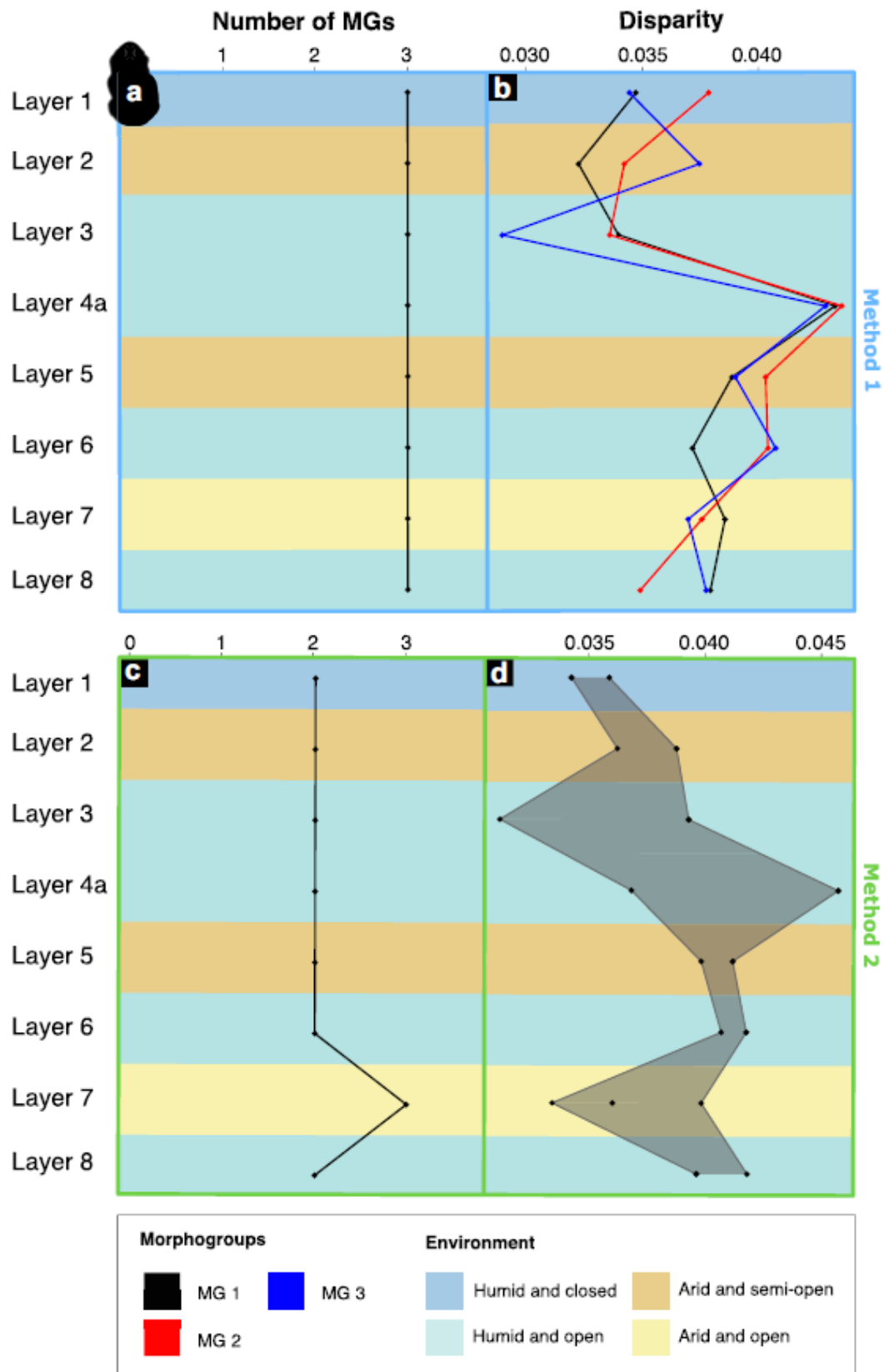


Fig. 5. Number of MGs and disparity of m1 (lower first molar) shape over the eight studied stratigraphic layers of EH2. Environmental conditions (Stoetzel 2009; El Hajraoui et al. 2012) are indicated by background colors. **a** Number of MGs obtained with method 1; **b** disparity of MGs of method 1; **c** Number of MGs obtained with method 2; **d** disparity of MGs of method 2.

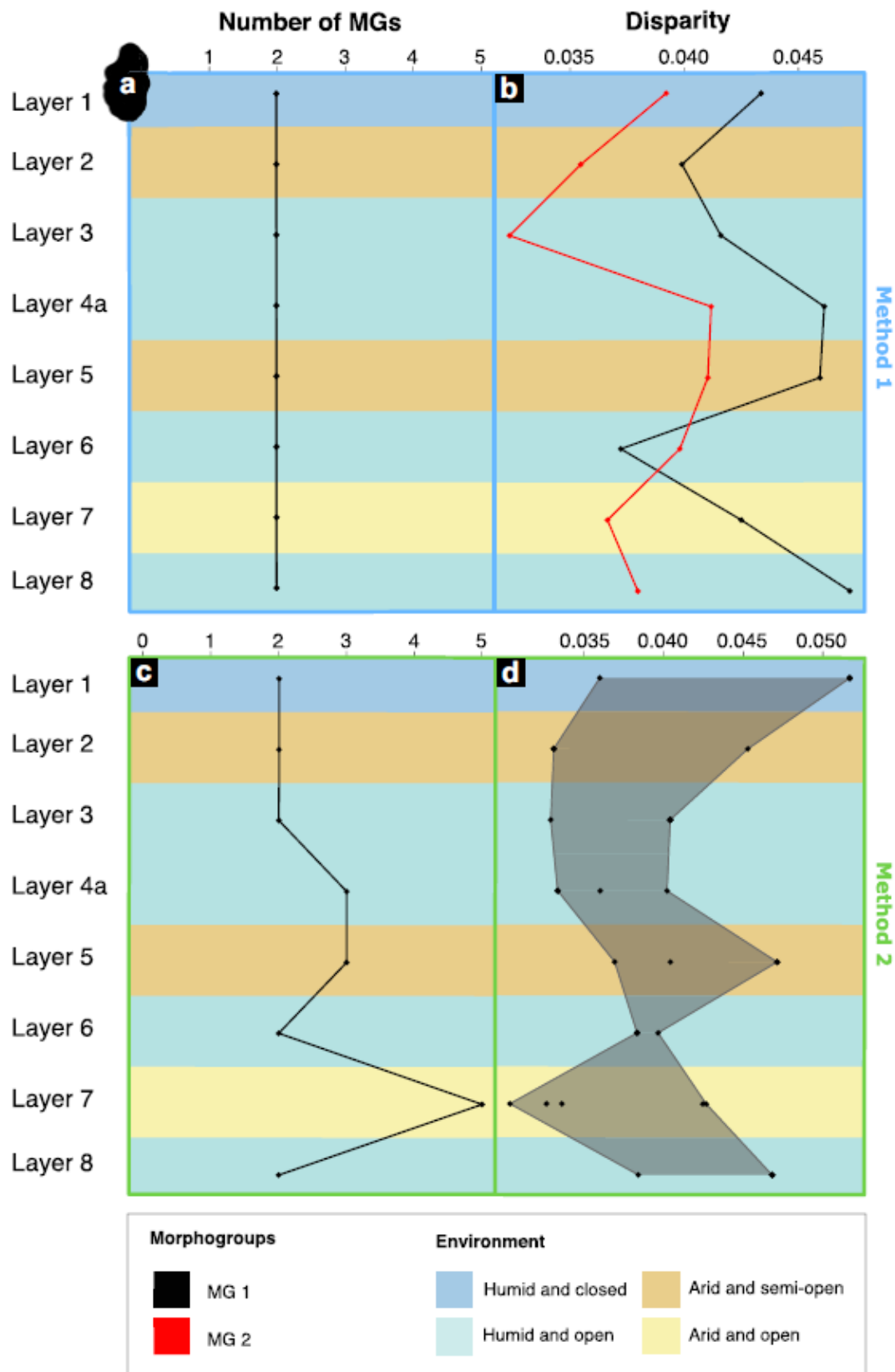


Fig. 6. Number of MGs and disparity of M1 (upper first molar) shape over the eight studied stratigraphic layers of EH2. Environmental conditions (Stoetzel 2009; El Hajraoui et al. 2012) are indicated by background colors. **a** Number of MGs obtained with method 1; **b** disparity of MGs of method 1; **c** Number of MGs obtained with method 2; **d** disparity of MGs of method 2.

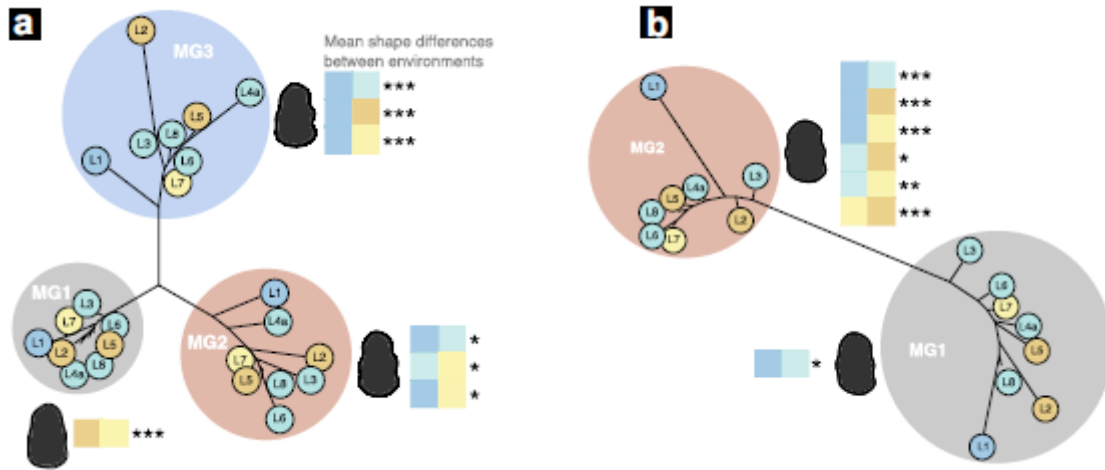


Fig. 7. Neighbor joining distance tree of the mean shape of MGs of method 1. Each layer of EH2 is referred as follows: "layer 1" is "L1". Environmental conditions (Stoetzel 2009; El Hajraoui et al. 2012) are indicated by background colors. **a** Neighbor joining distance tree between mean shape of MGs per layer for m1 (lower first molars) and results of pairwise Hotelling's T2 tests between environmental types per MGs; **b** Neighbor joining distance tree between mean shape of MGs per layer for M1 (upper first molars) and results of pairwise Hotelling's T2 tests between environmental types per MGs.

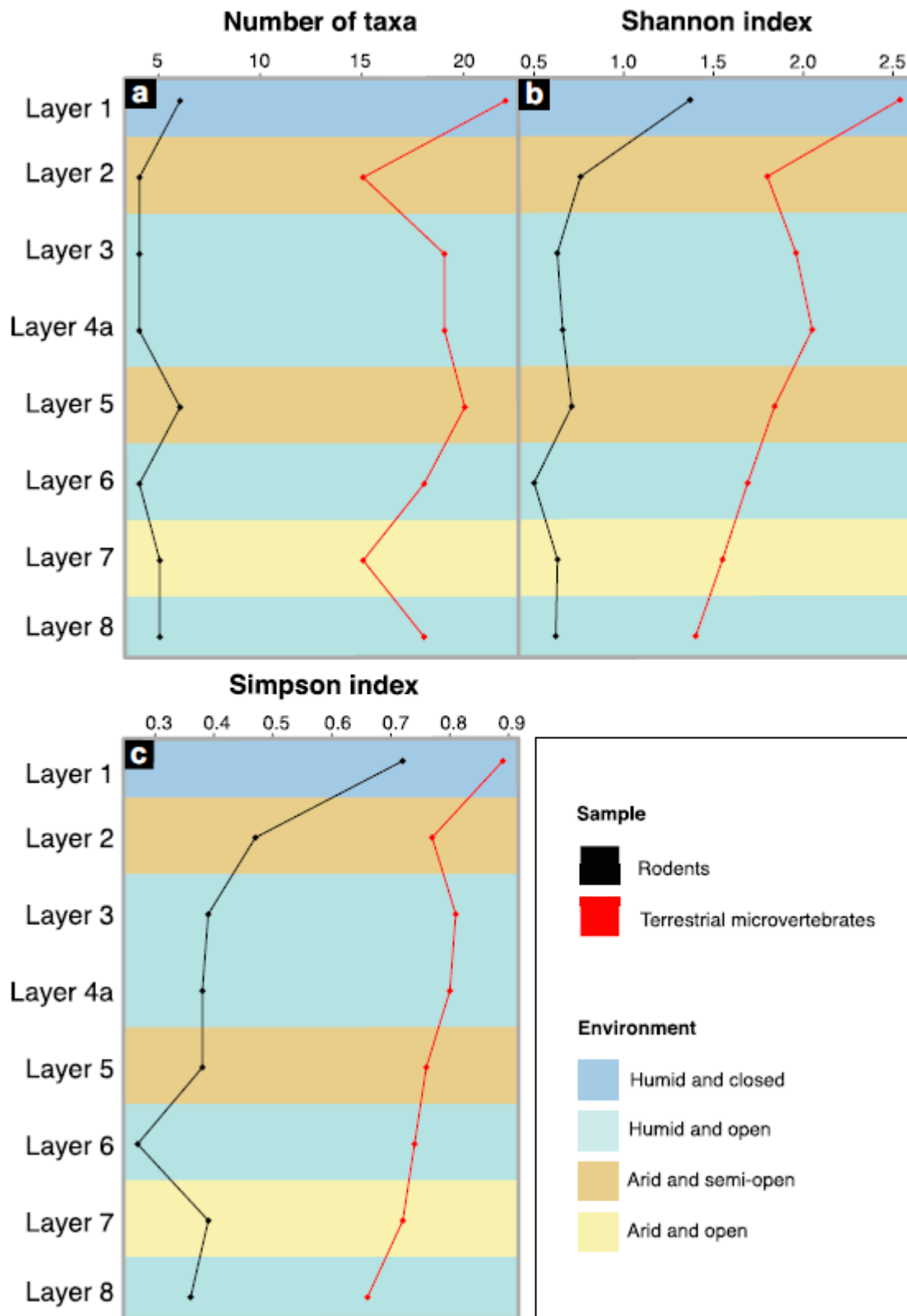


Fig. 8. Biodiversity indices over the eight studied stratigraphic layers of EH2. Environmental conditions (Stoetzel 2009; El Hajraoui et al. 2012) are indicated by background colors. **a** Number of taxa; **b** Shannon index; **c** Simpson index.

TABLEAUX

Layer	m1	M1
L1	74	69
L2	45	43
L3	64	65
L4a	57	50
L5	157	118
L6	189	181
L7	314	227
L8	233	237
TOTAL	1133	990

Table 1. Number of studied molars per layer of EH2. M1: upper first molars; m1: lower first molars.

		m1	M1
Method 1	Number of clusters	3	2
	K-values	399	500
	SI means	0.15	0.19
Method 2	Number of clusters	up to 3	up to 5
	K-values	22 < K < 140	13 < K < 84
	SI means	0.14 < SI < 0.29	0.10 < SI < 0.20

Table 2. Synthesis of clustering results of method 1 (one general clustering performed) and method 2 (one clustering performed per layer). M1: upper first molars; m1: lower first molars.