



Morphological and dietary adaptations to different socio-economic systems in Chalcolithic dogs

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ABSTRACT

Dogs have cohabited with humans since the Upper Paleolithic and their lifestyle and diet during late prehistory probably already depended on the role they played in past societies. Here, we used a combination of stable isotope analyses and three-dimensional geometric morphometrics to test for differences in, and associations between, diet and mandibular morphology based on 150 dogs of three sites of the Chalcolithic Gumelnița culture in Romania (4550–3900 cal. BCE) characterized by different socio-economic systems. At Hârşova-tell and Boruşani-Popină, where the subsistence economy is mainly based on herding, dogs have a variable diet that is rich in domestic animals (sheep, cattle, pig) and may contain fish. In contrast, at Vităneşti-Măgurice, where hunting predominates, the diet of dogs is more specialized towards large game (red deer, aurochs, wild horse), reflecting the composition of human food refuse. Moreover, dogs have more robust (but not larger) mandibles at this site with shapes suggesting a greater importance of the temporal muscle important for the capture of large prey and the breaking of large bones. The strong covariation between mandible shape and stable isotope signatures suggests functional adaptations to diet. Overall, our results support the idea that prehistoric dogs adapted to human lifestyles.

1. Introduction

In Europe, dogs have been integrated within human groups at least since the Magdalenian period, circa 17,000 years ago (Boudadi-Maligne et al., 2020; Hervella et al., 2022; Janssens et al., 2018). From the Mesolithic onwards, they played a diversity of roles, as they were subjected to social or symbolic practices, were used as food supply (Grandal-d'Anglade et al., 2019; Horard-Herbin et al., 2014; Larsson, 1994), and were also probably allies in hunting activities and served for the protection of camps or villages. Accordingly, they were in the foreground of changes in subsistence strategies or cultural practices which may have influenced their access to food as well as their lifestyle (e.g.

Arendt et al., 2016; Grandal-d'Anglade et al., 2019; Ollivier et al., 2016). This may have left imprints on dog skeletal remains involving both the stable isotopic signature reflecting diet (Grandal-d'Anglade et al., 2019; Laffoon et al., 2015; Schulting and Richards, 2002) and jaw shape which is determined in part by muscle forces and prey properties (Brassard et al., 2020c; Neaux et al., 2022; Nogueira et al., 2009).

Several previous studies have used three-dimensional (3D) geometric morphometrics to explore the morphological diversity of ancient canids in North America or Russia (Ameen et al., 2017; Drake et al., 2015, 2017; Ledoux and Boudadi-Maligne, 2015; Losey et al., 2022a). Yet, only a few have focused on European Neolithic dogs (Brassard et al., 2022). We previously demonstrate that, in Europe, dogs exhibit an

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important range of morphological variability in mandible shape and size as early as the Neolithic and Chalcolithic (Brassard et al., 2022). The lower jaw of modern and ancient dogs was found to differ in functionally important areas, possibly reflecting differences in diet, competition, or the implication of ancient dogs in hunting or defense. However, whether and how this diversity in mandible shape is related to specific environmental or anthropogenic factors remains largely unexplored. In southern Romania, a great number of dog remains – especially mandibles – were found in settlements from the Late Chalcolithic Gumelnița culture, dated from *circa* 4550–3900 cal. BCE (Bălășescu, 2014; Bălășescu et al., 2005a, 2005b; Lazăr et al., 2016). The chrono-cultural context and subsistence economy of human communities at these sites are well-documented, offering the opportunity to better understand the morphological variability in dogs in Eastern Europe at that time. In particular, the Gumelnița culture is characterized by an important diversity in animal exploitation systems in comparison with preceding Chalcolithic cultures (Bréhard and Bălășescu, 2012). While subsistence economies rely on cattle and sheep/goat husbandry during the Early Chalcolithic, large game played a major role in some communities of the Gumelnița, and a great variability is observed among both the preferentially hunted and reared mammals (i.e., cattle, sheep/goat or pig) species. These differences among Gumelnița communities possibly impacted the diet and role of dogs as well as their morphology. Given that the mandible must resist the forces generated during biting, the consumption and/or hunting of different food items likely imposes different selective pressures on mandible shape that may reflect these different contexts (Karamani et al., 2022; Neaux et al., 2022).

In this study we focused on three geographically and chronologically close sites: Hârșova-tell, Bordușani-Popină and Vitănești-Măgurice (Fig. 1). Despite the fact that these sites all relate to the Gumelnița culture and are partly contemporary, significant differences in their socio-economic systems have been described previously. Whereas the animal exploitation system mostly relies on husbandry at Bordușani-Popină and Hârșova-tell (although a substantial part of the subsistence was also derived from aquatic resources, i.e. fish and bivalves), wild taxa predominate the mammal assemblage (up to 61%) at Vitănești-Măgurice (Bălășescu et al., 2005a, 2005b; Bréhard and Bălășescu, 2012) (Fig. 1, SI 1). The greater importance of hunting activities at Vitănești-Măgurice, which focused on large game such as red deer, aurochs, wild boar, and

wild horse (Fig. 1, SI 1) may have had repercussions on dogs. Indeed, the dogs from Vitănești-Măgurice were likely more involved in hunting activities, and the proportion of large game in their diet may have been more important. The lower proportion of dog remains in the mammal assemblage of Vitănești-Măgurice compared to Bordușani-Popină and Hârșova-tell (Fig. 1; SI 1) supports the assumption that dogs had different roles/tasks. At all sites dog carcasses were, however, skinned, dismembered, and used for consumption as attested by numerous cuts and localized burn marks observed on the bones (Lazăr et al., 2016) (SI 2). In addition, preliminary stable isotope analyses performed on dog bone collagen (Balasse et al., 2016, 2017) suggest differences in diet as attested by the lower stable nitrogen isotope values for the dogs from Vitănești-Măgurice.

To test whether dogs exhibit differences in diet and morphology between Gumelnița sites characterized by either predominant hunting (Vitănești-Măgurice) or husbandry (Hârșova-tell and Bordușani-Popină), we used two complementary approaches, stable isotope analyses and geometric morphometrics. Many studies have explored ancient dogs diet through stable isotope analyses in a variety of contexts (e.g. Drake et al., 2017; Grandal-d'Anglade et al., 2019; Laffoon et al., 2015; Losey et al., 2022b; Schulting and Richards, 2002). Additionally, as mentioned above, 3D geometric morphometrics has been proven to be helpful to describe morphological variability in ancient canids/dogs (Brassard et al., 2022; Drake et al., 2015, 2017; Losey et al., 2022a). However, stable isotope analyses and GMM are rarely combined in dogs (Drake et al., 2017; Losey et al., 2022b). Although the combination of these approaches can bring new and complementary insights into the adaptation of species to their environment (Balasse et al., 2016; Barberena et al., 2020; Cucchi et al., 2016), in particular by testing the covariation between mandibular morphology and the stable isotope signature. Stable carbon and nitrogen isotope ratios ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) in bone collagen can be used to test the contribution of different dietary sources, provided that these are isotopically distinct. Previous studies on the faunal remains from Vitănești-Măgurice, Bordușani-Popină and Hârșova-tell showed differences between the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values measured in three large animal categories present in the subsistence economy: domestic mammals, wild terrestrial mammals, and fish (Balasse et al., 2016, 2017). At all sites, fish had generally lower $\delta^{13}\text{C}$ values compared to terrestrial fauna and the highest $\delta^{15}\text{N}$ values in the

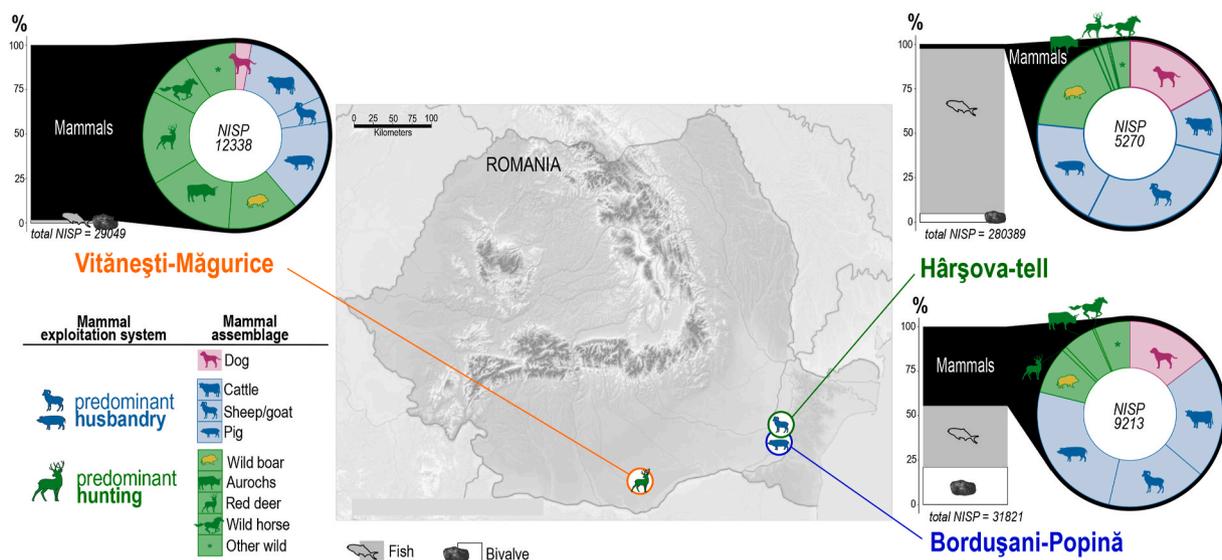


Fig. 1. Location of the three Romanian archaeological sites of the Late Chalcolithic Gumelnița culture considered in this study, with bar and pie charts representing the relative proportion of mammals and aquatic resources (i.e. fish and bivalves) in the faunal assemblages at each site (“total NISP”), and of the main taxa identified among the mammal assemblages (see SI 1 for raw data). The color of each site is used as a color code in the following figures. Different symbols indicate the main mammal exploitation system. Other wild: roe deer, wild carnivores, and beaver. NISP: number of identified specimens. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

entire food network (in case of piscivorous fish). Moreover, domestic herbivores (cattle and sheep) yielded higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values than wild herbivores (aurochs, red deer, roe deer, wild boar and wild horse). Consequently, the respective contribution of these three main items in the diet of dogs can be investigated using their bone collagen $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values.

In parallel, geometric morphometrics is commonly used to describe bone shape allowing to quantify morphological diversity (e.g. Brassard et al., 2022; Drake et al., 2017, 2015; Losey et al., 2022a). In this study we focused our analyses on the shape of the lower jaw (i.e., the mandible). Not only is it well preserved in archaeological faunal assemblages, but in modern dogs it has been proven to be informative on the overall shape of the head (Brassard et al., 2020b) and masticatory function through its relation with muscle size and position, and bite force (Brassard et al., 2020a, 2020c). The shape of the mandible is not only determined by genetics but it is also shaped by environmental and anthropogenic constraints (phenotypic plasticity), as previously demonstrated in other taxa (e.g. Anderson et al., 2014; Neaux et al., 2022). By performing geometric morphometrics analyses on all mandibles for which stable isotopic data are available, we can quantitatively test for a link between morphology and diet. This may in turn provide information on the adaptation of contemporary dogs to different living conditions according to the socio-economic system.

2. Material and methods

2.1. Archaeological sites under study

Hârşova-tell (Constanţa County) and Borduşani-Popină (Ialomiţa County) are located approximately 20 km apart, on the Danube River Plain in South-Eastern Romania. The Borduşani-Popină village was established on the large islands of Balta Ialomiţei and Hârşova-tell on the eastern side of the river. Vităneşti-Măgurice (Teleorman County) is located around 250 km further west, in the Teleorman river floodplain in the middle South of the country.

2.2. Cultural attribution and dating

In this study, we only considered the animal remains which are related to the Gumelniţa occupation of these settlements. Dogs from these sites are sub-contemporary, since the Gumelniţa occupation is radiocarbon dated to circa 4500–4250 cal. BCE at Borduşani-Popină (Bréhard et al., 2014; Gillis et al., 2013), circa 4350–4050 cal. BCE at Hârşova-tell (Bréhard and Bălăşescu, 2012), and circa 4480–4000 cal. BCE at Vităneşti-Măgurice (see SI 3).

2.3. Socio-economic systems

Important differences in the socio-economic systems exist between the three sites during the Gumelniţa occupation. When considering mammals, the subsistence economy mostly relies on husbandry at Borduşani-Popină and Hârşova-tell with 79% and 77% of the identified mammal remains being domestic (Fig. 1, SI 1). At Borduşani-Popină, domestic pigs predominate the number of remains, followed by domestic cattle, sheep/goats, and dogs (see synthesis in (Bréhard and Bălăşescu, 2012)) (SI 1). At Hârşova-tell, domestic sheep/goats dominate during the Gumelniţa occupation, followed by domestic pigs, dogs, and cattle. In contrast, at Vităneşti-Măgurice, hunting was very important during the Gumelniţa: wild taxa, mostly red deer, aurochs, wild boar, and wild horses, represent 61% of the mammal remains (Fig. 1, SI 1). Moreover, aquatic resources were collected in large quantities during the Gumelniţa at Hârşova-tell and Borduşani-Popină. Based on the total number of identified specimens (total NISP), fish represent 93% and 34% of the total archaeozoological remains, respectively, bivalves represent 5% and 21%, and mammals 2% and 45% (Bălăşescu et al., 2005b) (Fig. 1). At Vităneşti-Măgurice, these resources probably played

a less important role as fish remains represent less than 1%, bivalves 1%, and mammals 98% of the remains. The very low relative proportion of fish at this site should, however, be taken with caution as sediments have not been sieved during excavations at this site, contrary to Hârşova-tell and Borduşani-Popină.

The relative proportion of dog remains is very high in the faunal assemblages of Borduşani-Popină and Hârşova-tell during the Gumelniţa occupation (15% and 17% of mammal remains, respectively), compared to Vităneşti-Măgurice (3%; Fig. 1, SI 1). At the three sites, dog bones are fragmented and were found mixed with bones from other taxa in areas of household refuse. Numerous cuts and localized burn marks were observed on dog bones, attesting that carcasses were skinned and dismembered, and that the meat was eaten (Lazăr et al., 2016) (SI 2).

Clear evidence of bone gnawing by dogs (or other carnivores) and pigs has been described at the three sites (the number of bones gnawed ranges from 8% to 18% of the total mammal remains, Bălăşescu unpublished), proving that dogs had access to carcasses at the three settlements.

2.4. Material

The study material used for the shape analysis is composed of 77, 46, and 27 dog (*Canis familiaris*) mandibles from Borduşani-Popină, Hârşova-tell, and Vităneşti-Măgurice respectively (see details about the sample in SI 4). For preliminary comparison of mandible sizes, we also considered mandibles of modern ($n = 8$) and ancient wolves ($n = 7$, including 3 from Vităneşti-Măgurice) that were previously studied in Brassard et al. (2022). Dogs were primarily distinguished from wolves based on their mandibular size (see Brassard et al., 2022; see SI 6). To limit the effect of ontogeny, only dentally mature specimens with the first molar completely erupted were considered for geometric morphometric analyses. Among those, a subsample of 19 dogs from Borduşani-Popină, 17 dogs from Hârşova-tell, and 26 dogs as well as two foxes (*Vulpes vulpes*) and three wolves (*Canis lupus*) from Vităneşti-Măgurice were selected for stable isotope analysis. These newly acquired data complement previously published data from five dog mandibles from each site (Balasse et al., 2016). Right and left mandibles were included to increase the sample. At each site, we checked that they belong to different individuals (Minimum Number of Individuals) by visually examining the global morphology, and by comparing the 3D models and stable isotope signatures.

2.5. Bone collagen extraction and stable isotope analysis

Bone was sampled by sawing. After bone external surfaces and the spongiosa were removed by drilling, the bone was mechanically reduced to powder and only the 355–710 μm fraction was used for collagen extraction. Collagen was extracted from about 200 to 300 mg of this powder following the procedure described by Bocherens et al. (1991), except for the final solubilization step which was performed at 70 °C. Coupled measurements of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were conducted on approximately 350 μg of collagen on an elemental analyzer (Thermo Flash, 2000) interfaced to a Thermo DeltaV Advantage IRMS. The analytical precision, determined by six to 12 analyses of our laboratory standard (alanine) within each run, varied from 0.05‰ to 0.14‰ for $\delta^{13}\text{C}$ and from 0.10‰ to 0.16‰ for $\delta^{15}\text{N}$, and from 0.20% to 0.35% for C content and from 0.07% to 0.13% for N content. Over the course of the analyses the alanine standard gave mean values of $+0.75 \pm 0.13\text{‰}$ for $\delta^{15}\text{N}$ ($N = 40$, expected value = $+0.59\text{‰}$), $-21.57 \pm 0.17\text{‰}$ for $\delta^{13}\text{C}$ ($n = 30$, expected value = -22.16‰), $15.60 \pm 0.19\%$ for N content ($N = 24$, expected value = 15.72%) and $40.36 \pm 0.59\%$ for C content (expected value = 40.44%). The stable isotope ratios were corrected accordingly using the Marbre LM average values for each analysis session. We compared the $\delta^{15}\text{N}$ values of dogs between the three sites using a Wilcoxon-Mann Whitney rank test. At each site, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values measured in dogs were further compared to those previously measured

in the associated fauna to provide broad guidance on the composition of the diet in dogs. Yet, this does not allow to account for the relative weight of the different isotopic items. Data from Borđuşani-Popină and Hârşova-tell were considered together as the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values measured in the different species at both sites do not differ significantly (Balasse et al., 2017).

2.6. 3D modelling of mandibles and geometric morphometric analyses

Three-dimensional numeric models of the mandibles were obtained using photogrammetry. One hundred photographs were taken from different angles while turning around the bone. The photos were aligned using ‘Agisoft PhotoScan’ (©2014 Agisoft LLC, St Petersburg, Russia). Where needed, the models were repaired (windows corresponding to extractions for DNA samples were filled in), cleaned, simplified, and mirrored (all mandibles were transformed into a ‘right’ mandible) using © ‘Geomagic Wrap’ 2013.0.1.1206 and ‘MeshLab’ v 2020.03 (Cignoni et al., 2008).

Three-dimensional landmarks were placed on the 3D models using the ‘Landmark’ software, version 3.0.0.6 (©IDAV, 2002–2005) (Wiley et al., 2005). Three landmarking protocols were considered based on the fragmentation of the material (Fig. 2, sample sizes for each protocol are reported in Table 1; see SI 4 for a listing of the mandibles included in each protocol). The first protocol is the most representative of the overall shape of the mandible, with a total of 388 landmarks (including 23 anatomical landmarks, 190 sliding semi-landmarks on curves, and 175 sliding semi-landmarks on the surface). Yet, whereas this protocol represents the sites of Borđuşani-Popină and Hârşova-tell well, the mandibles from Vităneşti-Măgurice are mostly too fragmented to be included in the analyses with this protocol ($n = 2$). To provide better comparisons with dogs from Vităneşti-Măgurice, we used two other protocols adapted to fragmented mandibles. The second protocol is the most representative of the shape of the mandibular body, with a total of 121 landmarks (including 14 anatomical landmarks, 40 sliding semi-landmarks on curves, and 67 sliding semi-landmarks on the surface). It allows to increase the sample size for Vităneşti-Măgurice ($n = 14$), and thus to consider this site. The third protocol is a subsample of the first protocol and only represents a very small part of the mandibular body at the junction with the mandibular ramus (involving only six anatomical landmarks and 20 sliding semi-landmarks on curves). This last protocol is less representative of the overall shape of the mandible, but allows to incorporate more mandibles in the analyses ($n = 150$), in particular mandibles from Vităneşti-Măgurice which are often fragmented ($n = 27$, see sample details in Table 1).

All statistical analyses were run in ‘R’ version 4.0.0 (2020-04-24) (R Core Team, 2021). The sliding semi-landmarks were slid and transformed into spatially homologous landmarks using a sliding semi-

Table 1

Results of the analyses to compare mandible shape and size between sites. BOR: Borđuşani-Popină; HAR: Hârşova-tell; VIT: Vităneşti-Măgurice; P: p-value; R^2 : coefficient of determination; CV: success rate of the cross-validation; n PC: number of principal components considered in analyses; PV: Procrustes variance. Significant differences are in bold.

	Protocol 1	Protocol 2	Protocol 3
Sample size	28 BOR, 14 HAR, 2 VIT	66 BOR, 35 HAR, 14 VIT	77 BOR, 46 HAR, 27 VIT
Morphological disparity	p(BOR-VIT) 0.040 PV _{HAR} 0.0036 PV _{BOR} 0.0034 PV _{VIT} 0.0059	p(BOR-VIT) 0.080 PV _{HAR} 0.0044 PV _{BOR} 0.0044 PV _{VIT} 0.0062	p(BOR-VIT) 0.031 PV _{HAR} 0.0098 PV _{BOR} 0.010 PV _{VIT} 0.017
Difference in mean shape (Procrustes ANOVA)	P = 0.001, $R^2 = 0.12$ HAR-BOR 0.005 HAR-VIT 0.005 BOR-VIT 0.001	P < 0.001, $R^2 = 0.063$ HAR-BOR 0.008 HAR-VIT 0.10 BOR-VIT 0.001	P < 0.001, $R^2 = 0.12$ HAR-BOR 0.003 HAR-VIT 0.009 BOR-VIT 0.001
Difference in mean size (ANOVA)	P = 0.3	P = 0.14	P = 0.8
KNN (set.seed(100))	k 8 CV 82% BOR 29% HAR 100% VIT	8 74% BOR 34% HAR 14% VIT	6 75% BOR 37% HAR 19% VIT
CVA - PCA 97% of variance	n PC CV 100% BOR 100% HAR 100% VIT	PC 1 to 25 89% BOR 69% HAR 86% VIT	PC 1 to 9 81% BOR 46% HAR 44% VIT

landmark procedure minimizing the overall bending energy, implemented in the ‘Morpho’ package (version 2.9) in R (Schlager, 2013). To decompose the mandible form into centroid size (a proxy of the overall size of the object) and shape (which represents the proportions based on the differences between the different landmarks), we performed a Generalized Procrustes analyses (GPA) (Goodall, 1991) using the function ‘procSym’ (Dryden and Mardia, 2016; Gunz et al., 2005; Klingenberg et al., 2002) from the package ‘Morpho’. A well-preserved mandible was warped to the consensus of the GPA using thin-plate spline deformation for all further visualizations (Klingenberg, 2013).

To test for differences in mean mandible centroid size, we performed ANOVAs using the function ‘aov’. To compare morphological disparity (i.e. variability) in mandible shape between sites we performed a

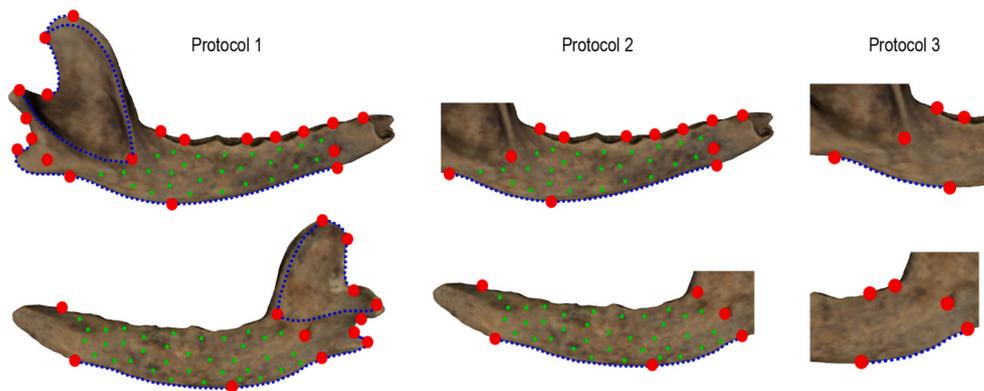


Fig. 2. Landmarking protocols according to the different fragmentation patterns. Different types of landmarks are represented by different colors: anatomical landmarks are in red, sliding semi-landmarks on curves are in blue, sliding semi-landmarks on surfaces are in green. Sample sizes for each protocol are reported in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

disparity test using the function ‘morphol.disparity(coords ~ 1, groups = ~ site, iter = 999)’ in the package ‘geomorph’ (Foote, 1993; Zelditch et al., 2012). This function estimates the morphological disparity for each group (which is the Procrustes variance within each group without covariates and using the overall mean of the sample, the value being adjusted by the sample size for each group) and performs pairwise comparisons to identify differences among groups. To test for differences in mean mandible shape, we performed Procrustes ANOVAs and post-hoc tests, using the functions ‘procD.lm(coords ~ site, iter = 999)’ and ‘pairwise’, respectively (Adams and Collyer, 2016, 2017; Anderson and Braak, 2003; Anderson, 2001; Collyer et al., 2015; Goodall, 1991). Differences between sites were visualized using Canonical Variate Analysis (CVA), which maximizes among-group variances relative to within-group variances (Campbell and Atchley, 1981; Klingenberg and Monteiro, 2005). CVA was performed on the scores of the first Principal Components to reduce the number of variables (representing 97% of the total variability in shape). To visualize shape deformations along the canonical axes, we calculated the theoretical minimum and maximum shapes for each axis, and visualized the deformation from the mean shape of the total sample towards these theoretical shapes (for visualization purposes deformations were amplified by a factor 2) using functions ‘restoreShapes’ and ‘plotRefToTarget’. Considering that the effectiveness of the CVA to discriminate groups is strongly dependent on the ratio between the number of variables and sample size (Mitteroecker and Bookstein, 2011) we also performed k-Nearest Neighbour Classification (k-NN) with a leave-one-out cross validation (using function ‘knn.cv’ from package ‘class’) (Ripley, 1996; Venables et al., 2002). k-NN is a non-parametric method that classifies an individual according to the a priori classification of its k neighbors (by Euclidean distance) using a majority vote. KNN is less sensitive to number of variables with respect to the sample size than the CVA. The k was determined using function knn.cv: we kept the k which led to the best cross-validation rate. Considering that the leave-one out procedure leads to different results each time the analyses are conducted, the results have to be considered as an approximate success rate.

We tested the relationship between isotopic signature ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and morphometric data (mandible shape) for a subsample of mandibles for which we had both types of data (sample size for each protocol is reported in Fig. 4). We explored the patterns of covariation between the two datasets using a Two-Block Partial Least Square regression (2B-PLS), using the function ‘pls2B’ (Rohlf and Corti, 2000). P-values were calculated based on 1000 permutations. Visualization at the minimum or maximum of the PLS axis was obtained using the functions ‘plsCoVar’, ‘tps3d’ and ‘deformGrid3D’.

3. Results

3.1. Bone collagen stable isotope analysis

Of the 67 bone powders, only one from Hârşova-tell (Har3) was lost due to complete dissolution during the initial demineralisation step with 1M HCl. One specimen from Vităneşti-Măgurice (Vit12) also had an unusual sandy aspect after freeze-drying and low C and N contents (13.8% and 5.0% respectively) and was excluded from the final dataset. Collagen extraction yielded an average of 71 mg/g at Borduşani-Popină (27–123 mg/g), 99 mg/g at Hârşova-tell (64–124 mg/g), and 82 mg/g at Vităneşti-Măgurice (23–127 mg/g) (SI 4). Carbon contents vary from 31% to 44%, nitrogen contents from 12% to 16% and atomic C:N ratios from 3.12 to 3.28, satisfying the criteria defined for reliable stable carbon and nitrogen isotope ratios (Ambrose, 1990; DeNiro, 1985; Guiry and Szpak, 2021).

The distribution of stable isotope values in dogs from the three sites are shown in Fig. 3 and reported in SI 4. Dogs $\delta^{13}\text{C}$ values overlap at the three sites, ranging from -23.0‰ to -18.8‰ . However, the dispersion in $\delta^{13}\text{C}$ values measured in dogs is considerably reduced at Vităneşti-Măgurice (coefficient of variation $\delta = 0.46\text{‰}$; from -21.2‰ and

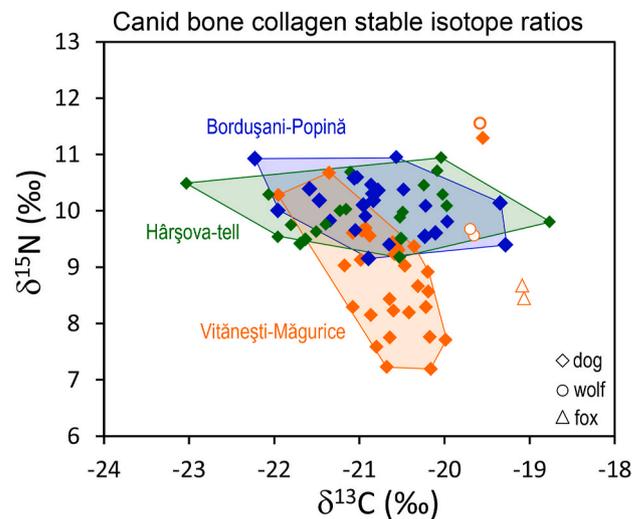


Fig. 3. Results from stable carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope analysis in canid bone collagen from Borduşani-Popină ($n = 24$), Hârşova-tell ($n = 21$) and Vităneşti-Măgurice (dogs = 31, wolves = 3 and foxes = 2). The dataset includes the data previously published in (Balasse et al., 2016).

-20‰), compared to Borduşani-Popină ($\delta = 0.70\text{‰}$; -22.2‰ to -19.3‰) and Hârşova-tell ($\delta = 0.96\text{‰}$; -23‰ to -18.8‰). At Borduşani-Popină and Hârşova-tell, dog $\delta^{15}\text{N}$ values vary within the same range (9.2‰–10.9‰). In contrast, the dogs from Vităneşti-Măgurice show a larger range (7.2‰–11.3‰) and globally lower $\delta^{15}\text{N}$ values, with a majority of them (14/25 or 56%) below the lowest values measured at Borduşani-Popină and Hârşova-tell ($<9.2\text{‰}$). When considering this new dataset with previously published data for dogs at the three sites (Balasse et al., 2016), the $\delta^{15}\text{N}$ values measured in the dogs from Vităneşti-Măgurice are significantly lower than those measured at Borduşani-Popină and Hârşova-tell considered together (Wilcoxon-Mann Whitney rank T-test, $W = 168.5$, $p = 4.10-8$). The two foxes at Vităneşti-Măgurice have higher $\delta^{13}\text{C}$ values compared to dogs (-19.1‰ each) and rather low $\delta^{15}\text{N}$ values (8.5‰ and 8.7‰), reflecting the significant contribution of fruits to their diet (Castañeda et al., 2020; Cavallini and Volpi, 1996). At Vităneşti-Măgurice, the three wolves have $\delta^{15}\text{N}$ values of 9.6‰–11.6‰, among the highest within terrestrial vertebrates at this site. It is noteworthy that one dog from Vităneşti-Măgurice (Vit38) differs from its counterparts at this site with higher values of $\delta^{13}\text{C}$ (-19.6‰) and $\delta^{15}\text{N}$ (11.3‰), more similar to those measured in one wolf, yet it is clearly a dog according to its mandibular size (Brassard et al., 2022).

When $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of dogs are compared to those of the associated fauna (Fig. 4) we observe that, at the three sites, dogs occupy the highest place in the terrestrial food web: they show the highest $\delta^{15}\text{N}$ values, only being surpassed by the wolf at Vităneşti-Măgurice. This place is shared with the pigs at Vităneşti-Măgurice, and to a lower extent at Borduşani-Popină and Hârşova-tell. Most $\delta^{15}\text{N}$ values measured in Vităneşti-Măgurice for wild and domestic herbivores do not differ significantly from those measured at Borduşani-Popină and Hârşova-tell (SI 5) except for the red deer, which shows slightly lower values at Vităneşti-Măgurice ($\delta^{15}\text{N} = 5.5 \pm 0.3$, versus 6.0 ± 0.5 at Borduşani-Popină and Hârşova-tell). More significantly, at the three sites, globally lower $\delta^{15}\text{N}$ values are measured in large wild herbivores (red deer, aurochs and wild horse) compared to domestic animals (cattle, sheep and pig) (Fig. 4). In particular, the wild horse yielded the lowest $\delta^{15}\text{N}$ values of the dataset (average: 3.9‰ and 4‰ respectively at Vităneşti-Măgurice and Borduşani-Popină - Hârşova-tell). Moreover, at Borduşani-Popină and Hârşova-tell, the global faunal spectrum occupies a wide range of $\delta^{13}\text{C}$ values (-25.5‰ to -15.4‰), including two sources of primary importance in the human subsistence: aquatic resources (pelagic fish; carp in particular) contributing with the lowest $\delta^{13}\text{C}$ values

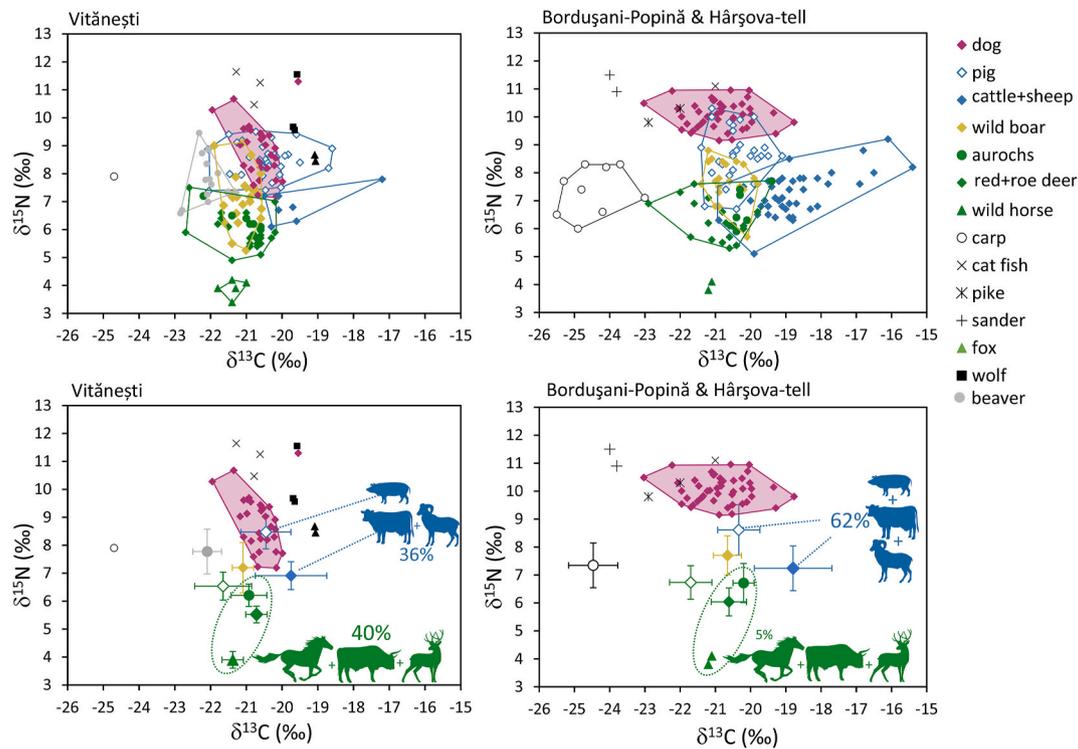


Fig. 4. Comparison between the stable carbon ($\delta^{13}\text{C}$) and nitrogen isotope values ($\delta^{15}\text{N}$) measured in dog bone collagen with the values previously measured in other species from the same assemblages (Balasse et al., 2016, 2017). A: Projection of all data. B: Average value of stable isotopes data per group for all species except dogs, and the relative proportion of each group in the mammal assemblages (in % of NISP; cf. Fig. 1). Detailed results for each specimen are reported in SI 4.

and cattle and sheep with the highest $\delta^{13}\text{C}$ values (Fig. 4). The $\delta^{13}\text{C}$ values measured in dogs are intermediate.

3.2. Morphological differences between dogs from different sites

For each of the protocols, no significant differences in size were

evidenced between sites (Table 1). Based on protocol 3 dog mandibles are much smaller and do not overlap those of modern and ancient wolves for all three sites (SI 6).

Analyses conducted with the smallest fragment (protocol 3) revealed, in spite of the small portion of mandible that it represents, that dogs from Vitănești-Măgurice have slightly more variable mandible

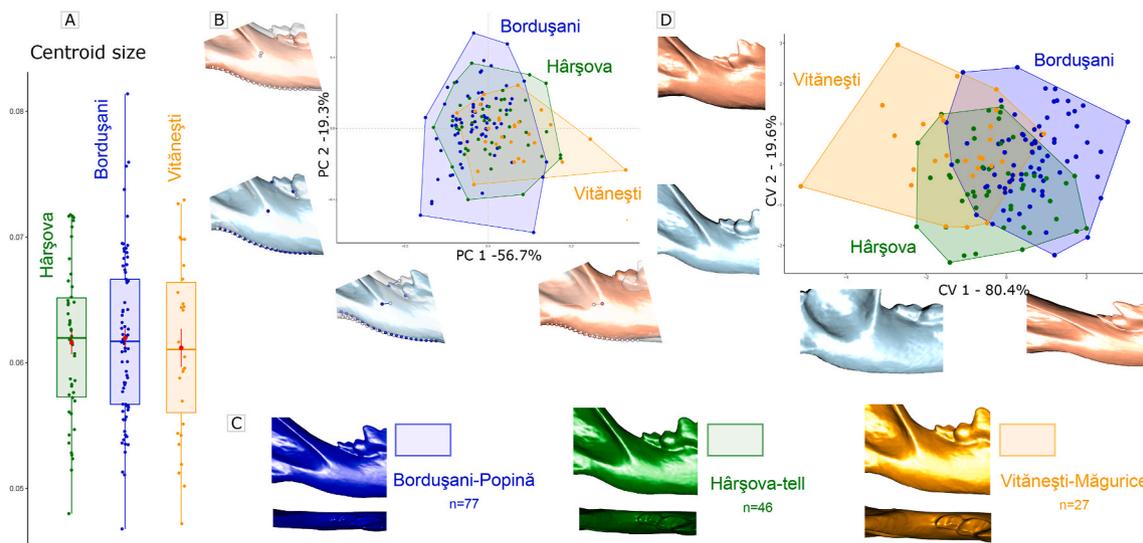


Fig. 5. Variability in mandible form in dogs from the Gumelnița culture and differences between the three sites, based on results from protocol 3. Dogs of the three sites (77 from Bordoșani-Popină, 46 from Hârșova-tell and 27 from Vitănești-Măgurice) are indicated by different colors. (A) Boxplots of the centroid size. See SI 6 for size comparison with modern and ancient wolves. (B) Principal Component Analysis on mandible shape. The theoretical shapes at the minimum and maximum of the PC axes (in blue and pink, respectively) are superimposed on the mean shape (white) and the vectors of deformation are reported. (C) Mean shapes for each site. (D) Canonical Variate Analysis on mandible shape. Theoretical shapes at the minimum and maximum of the CV axes are represented in blue and pink, respectively (the deformations from the mean shape are amplified by a factor 2). Results for protocols 1 and 2 are reported in the supplementary material, SI 7 and 8, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

shapes compared to dogs from the two other sites, and that the dogs of the three sites significantly differ in the mean mandible shape. The most important shape differences are between the dogs from Vitănești-Măgurice and those from Bordușani-Popină according to the Procrustes ANOVAs (Table 1), which is observable on the scatterplot representing the two first principal components. Some dogs from Vitănești-Măgurice have very high PC1 scores compared to the other dogs of our sample, and as such they are in a part of the scatterplot not occupied by dogs from Bordușani-Popină and Hârșova-tell (Fig. 5B). These dogs (which distinguish along PC1 in Fig. 5B and along CV1 in Fig. 5C) are characterized by a relatively more robust mandible. The rates of good classification in CVA and KNN are rather low (Table 1) due to strong overlap between sites. This is not surprising considering the reduced nature of this fragment, which is unlikely to reflect the total variation in mandible shape.

Analyses performed with the complete mandibular body (protocol 2; SI 6) also support the existence of shape differences between sites, with dogs in Vitănești-Măgurice having relatively more robust mandibles with a taller mandibular body. The rates of good classification are approximately the same for the KNN classification than for analyses with the smallest fragment. The description of the anatomical particularities of the dogs from Vitănești-Măgurice is supported by the visualizations made with the complete mandibles (protocol 1): the two robust mandibles from Vitănești-Măgurice, with a tall, large and backwardly inclined coronoid process, are clearly distinguishable from those from the two other sites on the right part of the PCA and along CV2 (SI 7). Additionally, analyses conducted with the complete mandibles show significant differences in the mean mandible shape between dogs from Hârșova-tell and Bordușani-Popină ($n = 14$ and $n = 28$, respectively).

According to the first axis of the CVA based on protocol 1 (SI 7), dogs from Bordușani-Popină have a taller mandibular body than in Hârșova-tell, with a ventral border of the mandibular body being more regularly curved; the coronoid process is more vertical and smaller, and the angular process is lower and larger. In dogs from Hârșova-tell, there is a pronounced curvature right under the carnassial tooth and the body goes straight upwards. Moreover, the coronoid process is oriented more backwards and is taller. The anatomical differences between dogs from Hârșova-tell and Bordușani-Popină are confirmed by analyses performed with protocols 2 and 3 (SI 6 and Fig. 5, respectively). For all protocols, the rates of good classification with the KNN method are similar and rather low, in particular for Hârșova-tell and Vitănești-Măgurice. This relatively low success rate suggests an important overlap between the three sites: only the most extreme shapes (mainly in Vitănești-Măgurice for protocol 2 and Bordușani-Popină for protocol 3) can be attributed with success to a site.

3.3. Relationship between stable isotope data and mandibular morphology

Significant covariations between mandible shape and stable isotope ratios are observed (Fig. 6). The results are statistically significant only for the protocols 2 and 3, which allow to conduct analyses on a sufficiently high sample size (Fig. 6B and C). The covariations are mostly driven by $\delta^{15}\text{N}$ values. Dogs with high $\delta^{15}\text{N}$ values (at the top right of the scatter plots), mostly from Bordușani-Popină and to a lesser extent from Hârșova-tell, are associated with slender mandibles with a smaller but more ventrally curved mandibular body. The bottom left of the scatterplot, only represented by specimens from Vitănești-Măgurice in protocols 2 and 3, associates dogs with low $\delta^{15}\text{N}$ values and robust mandibles with a tall mandibular body (Fig. 6B and C). Dogs from Vitănești-Măgurice are distributed throughout the scatterplot, whereas dogs from the two other sites are only present towards the top right for protocols 2 and 3.

4. Discussion

4.1. Reconstructing the diet of dogs at Bordușani-Popină, Hârșova-tell and Vitănești-Măgurice

The results from the stable isotope analyses demonstrate that at all sites dogs are positioned at the top of the food chain. However, we observed some differences between the dogs from Vitănești-Măgurice and those from the two other sites, which are partly due to the animal subsistence economy based on the hunting of large game at Vitănești-Măgurice (Fig. 1, SI 1 for details). Among the large game recovered, red deer (16% of the total remains and the best represented wild herbivore at Vitănești-Măgurice, versus only 6/2% at Bordușani-Popină/Hârșova-tell) and wild horses (8% of the mammal remains versus less than 1% at Bordușani-Popină and Hârșova-tell) are well represented. Their lower $\delta^{15}\text{N}$ values compared to domestic livestock, and the fact that they may potentially have contributed more to the diet of dogs, could partly explain why the dogs have lower $\delta^{15}\text{N}$ values at Vitănești-Măgurice compared to Bordușani-Popină and Hârșova-tell. Additionally, at Vitănești-Măgurice, higher $\delta^{15}\text{N}$ values were measured in wolves compared to dogs, suggesting a difference in diet composition. Whereas this may not necessarily be in terms of the nature of the prey it may imply differences in the relative proportions of prey with a lower proportion of large herbivores (aurochs and horse) in the diet of wolves. These species were targeted by the human community and therefore more readily available to the dogs at this site. The larger variation in $\delta^{13}\text{C}$ values in dogs from Bordușani-Popină and Hârșova-tell compared to Vitănești-Măgurice reflects the contribution of a diversity of resources

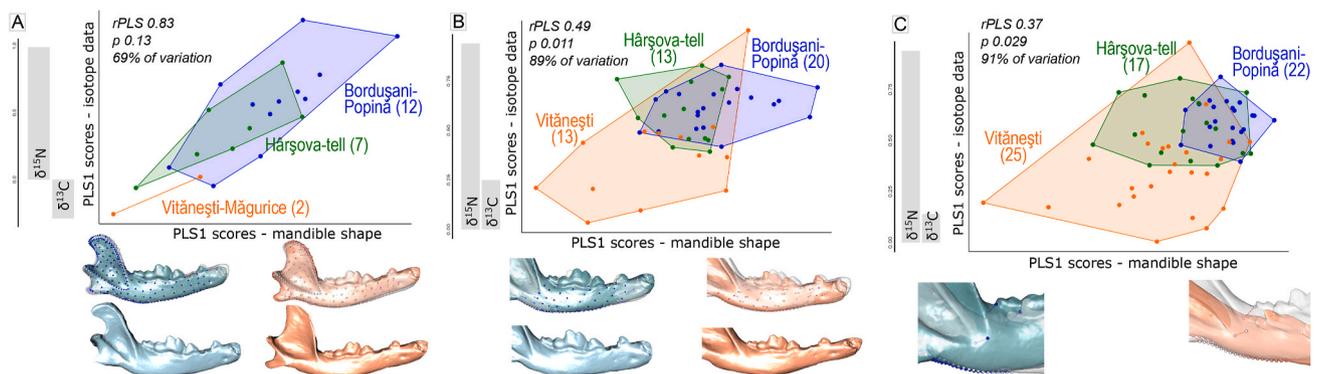


Fig. 6. Visualization of relationships between mandible shape and isotope data based on the 2-Block Partial Least Square Analysis performed using protocols 1 (A), 2 (B), and 3 (C). Vectors of isotope data ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) and shapes at the minimum and maximum of the PLS axis are represented. Top illustrations represent the deformations from the consensus to the extreme of the axis with the vectors of deformation in lateral view. Illustrations represent the shape at the extreme of the axis (amplified by a factor 2). The different sites are indicated by different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

to the diet of dogs rather than being specialized in one specific source (aquatic resources/protein from domestic stock). Yet, this also reflects greater interindividual variability in dog feeding behavior at these two sites with a more significant contribution of fish in the dogs with the lowest values (-22.2% and -23%) and a higher contribution of domestic cattle or sheep protein in the dogs showing the highest values (-19.3% to -19.8%). It is possible that the dogs from these sites had more autonomy (commensal lifestyle), resulting in a more varied diet. At Vitănești-Măgurice, the reduced range in $\delta^{13}\text{C}$ values reflects again a higher specialization in the consumption of protein from large game (Fig. 4). Interestingly, although dogs and pigs hold a similar position at the top of the food chain at Vitănești-Măgurice, possibly due to the consumption of human food waste by pigs (Balasse et al., 2016), the greater range in $\delta^{13}\text{C}$ values in pigs and a trend towards higher values suggest that some of the pigs clearly fed on a higher proportion of domestic animal protein compared to dogs, which were more specialized in large game.

4.2. Morphological variability in relation to diet

While the dogs from Vitănești-Măgurice had a diet more specialized in large game (especially wild horse and red deer as demonstrated by their lower $\delta^{15}\text{N}$ values and lower variability in $\delta^{13}\text{C}$ values), they also had relatively more robust mandibles (but of similar sizes) resulting in strong differences in the mean mandible shape compared to the two other sites (Fig. 5; Table 1). These morphological differences may be explained, at least partly, by different functional constraints imposed by the need to produce high bite forces at wide angles of jaw opening needed to crack bones of large mammals (Brassard et al., 2020a, 2020c, 2021; Turnbull, 1970). The link between anatomical traits and diet is supported by the significant and strong covariation between mandible shape and isotopic signature. The visualization of shape deformation along the covariation axis (Fig. 6) shows that dogs with lower $\delta^{15}\text{N}$ values present relatively more robust mandibles and with anatomical features (taller mandibular body) that suggest a greater importance of the temporal muscle (Turnbull, 1970). This muscle is particularly solicited to produce force at wide gape angles. Hence, the more powerful jaws of the dogs from Vitănești-Măgurice could have provided them with a selective advantage both during the hunting of large game and while breaking their bony remains.

The higher $\delta^{15}\text{N}$ values observed in dogs from Hârșova-tell and Bordușani-Popină suggest that they mostly fed on herded mammals (pig, sheep and cattle) of globally smaller size. Moreover, the greater variability in the diet in dogs from Hârșova-tell and Bordușani-Popină is accompanied by a reduced variability in mandible shape, likely because the consumption of fish does not exert strong mechanical constraints on the jaw. The observed generalist morphologies at these sites thus reflect the ability of these animals to cope with a diverse but not mechanically demanding diet. In addition, the mandibular morphology of dogs at Vitănești-Măgurice tends to be more variable than at Hârșova-tell and Bordușani-Popină, paralleling the greater variability in $\delta^{15}\text{N}$ values at this site. Along with the specialization towards large wild mammals (suggested by the lower variability in $\delta^{13}\text{C}$), this may illustrate the variability in the proportion of wild horse/red deer/aurochs in the diet of different dogs with dogs consuming more wild horse having the lowest isotopic values and the relatively most robust mandibles. This may suggest that different dogs were used to hunt different prey or that dogs had different roles or diets depending on their sex, for example. It is also possible that the preferentially hunted species varied through the five centuries of the Gumelnița occupation of the site; however, our dog sample size is too small to test this hypothesis.

Given that dogs are present in lower proportion in human food refuse at Vitănești-Măgurice compared to the two other sites (Fig. 1), we propose that dogs may have accompanied humans in hunting activities rather than being only a food resource. The morphology of the dogs from Vitănești-Măgurice could therefore be the result of either voluntary

selection by humans for certain morphologies of interest for hunting. Alternatively, involuntary selection by an adaptation to the way of life and the resources available could have shaped the mandibles of these dogs (the dogs could have fed on hunted prey as well as leftovers from carcass processing and human food scraps of mostly wild large mammals). It is also possible that these dogs, if they were used for hunting, were fed by humans with hunting leftovers to encourage them (e.g. Laporte, 2021; Plata et al., 2019), favouring the involuntary selection of robust jaws. Yet, the small size of the dogs (by comparison to modern or ancient wolves, see SI 6) makes it unlikely that they had the physical ability to catch and kill such large prey (in line with the hypothesis of Losey et al. (2022b)). Yet, they may have helped humans in hunting by running or driving down the game (as done by small modern hounds such as beagles), but strong bite force would not have been a particularly favored trait while hunting. Accordingly, the more likely explanation for their relatively more robust mandibular traits compared to the two other sites is that they fed on hunting leftovers and that the hard-to-chew and large diet items placed high functional demands on their masticatory apparatus, requiring relatively more strength when opening the jaw at a large gape (Herrel et al., 2008).

4.3. Morphological variability during the chalcolithic period

An important part of the variability in mandible shape in Hârșova-tell, Bordușani-Popină and Vitănești-Măgurice overlaps which may be related to the fact that the three populations share a same ancestral genetic pool (Ollivier et al., 2018), or similar lifestyles (Fig. 5B,D, S1B and S2B,D). Yet, we also evidenced some unique shapes allowing to distinguish the three sites. The most obvious differences separate Vitănești-Măgurice from the two other sites, reflecting how differences in the socio-economic system may have driven dog diversity in the past. However, significant differences were also observed in the morphology of the dogs from Bordușani-Popină and Hârșova-tell (which is particularly evident with complete mandibles, Fig. S1, Table 1). In particular, in Bordușani-Popină, some dogs are characterized by a taller mandibular body, a smaller and more triangular coronoid process and a lower angular process. These differences are observed despite the fact that the two dog populations are sub-contemporary and geographically very close, that the sites share similar characteristics regarding to the socio-economic system, and that humans consume dogs in large proportions in both settlements. These local differences are thus unlikely to be related to differences in the living conditions of dogs. Yet, the differences in mandible shape cannot be explained solely through genetic differences (plasticity renders things more complicated to decipher), they may at least partly reflect genetic drift or founder effects suggesting that exchanges of dogs between Hârșova-tell and Bordușani-Popină remained rare. This remains to be explored by paleogenetic approaches. If confirmed, this would also suggest that each settlement had its own population of dogs, and more widely that interbreeding would likely be even rarer with far more distant foraging communities.

5. Conclusion

Using a combination of isotopic and morphometric analyses, we identified differences in the diet and morphology of three sub-contemporary dog populations, dated to the same Chalcolithic culture and geographically close, but characterized by different socio-economic systems. At Vitănești-Măgurice, where the animal exploitation system is mainly based on hunting, dogs have a relatively more robust mandibular morphology (but of similar size), while their diet is more specialized in wild large mammals (red deer, aurochs, wild horse). This reflects adaptations to constraints imposed by their diet and possibly their role in hunting. In contrast, the dogs from Hârșova-tell and Bordușani-Popină have a diet that contains more domestic animals (sheep, cattle, pig), but also involves the consumption of fish in some individuals. Importantly, mandible shape reflects isotopic signatures in individual dogs suggesting

that diet is an important driver of the observed variation in mandible shape. Overall, dog mandibular morphology and diet appear to reflect human practices. The differences observed between the three sites reflect differences in the trophic level, and local adaptations to different diets depending on the living conditions. This illustrates the adaptation of dogs to different socio-economic systems as hypothesized.

Author contributions

Colline Brassard, Marie Balasse, Stéphanie Bréhard and Anthony Herrel designed research. Marie Balasse and Denis Fiorillo performed stable isotope data acquisition and analyses in the context of a project (CROC) lead by Morgane Ollivier. Marie Balasse performed analyses and interpretations of the stable isotopic data. Colline Brassard performed geometric morphometric (GMM) data acquisition and analyses. Colline Brassard, Stéphanie Bréhard and Anthony Herrel interpreted the GMM data. Adrian Bălăşescu, Stéphanie Bréhard and Valentin Radu provided archaeological samples of dogs for GMM analyses and detailed information regarding the archaeological contexts from which they were recovered. Colline Brassard, Marie Balasse, Stéphanie Bréhard and Anthony Herrel wrote the manuscript, with the scientific and editing input from Adrian Bălăşescu and Valentin Radu. All authors reviewed the manuscript and the supplementary information.

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Data availability

Detailed information about the sample and additional details of the results mentioned in the main text are provided in the electronic supplementary material. Three-dimensional models of the mandibles are available in the Dryad Digital Repository: <https://datadryad.org/stash/dataset/doi:10.5061/dryad.gqnk98sqd>. All data used for the morphometric analyses (3D coordinates of landmarks, R scripts and datasets) are available in SI 9.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jas.2023.105820>.

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