

1 This is **the unformatted final accepted version** of Hofman-Kamińska et al (2019) *Adapt*
2 *or die - response of large herbivores to environmental changes in Europe during the*
3 *Holocene* published in Global Change Biology.

4

5 The citation to the “online first” version should be:

6 Hofman-Kamińska E, Bocherens H, Drucker DG, Fyfe RM, Gumiński W, Makowiecki D, Pacher M,
7 Piličiauskienė G, Samojlik T, Woodbridge J, Kowalczyk R (2019: online first) *Adapt or die -*
8 *response of large herbivores to environmental changes during the Holocene* *Global Change*
9 *Biology* Doi:10.1111/gcb.14733

10

11 **Please check the permanent doi above for the final (published) definitive version on**
12 **the journal website and complete citation.**

13

38 **Keywords** *Alces alces*; aurochs; *Bison bonasus*; *Bos primigenius*; European bison; moose;
39 stable isotopes; ¹⁴C dating;

40

41 **ABSTRACT**

42 Climate warming and human landscape transformation during the Holocene resulted in
43 environmental changes for wild animals. The last remnants of the European Pleistocene
44 megafauna that survived into the Holocene were particularly vulnerable to changes in habitat.
45 To track the response of habitat use and foraging of large herbivores to natural and
46 anthropogenic changes in environmental conditions during the Holocene, we investigated
47 carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotope composition in bone collagen of moose
48 (*Alces alces*), European bison (*Bison bonasus*) and aurochs (*Bos primigenius*) in Central and
49 Eastern Europe. We found strong variations in isotope compositions in the studied species
50 throughout the Holocene and diverse responses to changing environmental conditions. All
51 three species showed significant changes in their $\delta^{13}\text{C}$ values reflecting a shift of foraging
52 habitats from more open in the Early and pre-Neolithic Holocene to more forest during the
53 Neolithic and Late Holocene. This shift was strongest in European bison, suggesting higher
54 plasticity, more limited in moose, and the least in aurochs. Significant increases of $\delta^{15}\text{N}$
55 values in European bison and moose are evidence of a diet change towards more grazing, but
56 may also reflect increased nitrogen in soils following deglaciation and global temperature
57 increases. Among the factors explaining the observed isotope variations were time (age of
58 samples), longitude and elevation in European bison, and time, longitude and forest cover in
59 aurochs. None of the analyzed factors explained isotope variations in moose. Our results
60 demonstrate the strong influence of natural (forest expansion) and anthropogenic
61 (deforestation and human pressure) changes on the foraging ecology of large herbivores, with
62 forests playing a major role as a refugial habitat since the Neolithic, particularly for European

63 bison and aurochs. We propose that high flexibility in foraging strategy was the key for
64 survival of large herbivores in the changing environmental conditions of the Holocene.

65

66 **INTRODUCTION**

67 The Holocene has been an epoch of highly dynamic environmental changes (Roberts *et al.*,
68 2018). Warming of the climate since the Pleistocene/Holocene transition and Late
69 Weichselian Glacial retreat in Europe resulted in forest expansion during the Early Holocene,
70 with maximum forest cover established by around 8200 cal yrs BP. Early Holocene (11,650
71 - 8200 cal yrs BP) (Walker *et al.*, 2012) tree migration and forest expansion was followed
72 by human expansion and the development of agriculture during the Neolithic (between 7000
73 to 2600 cal yrs BP, although dates for the Neolithic vary slightly in different regions of
74 Central and Eastern Europe) (Puhe & Ulrich, 2001). The start of the Neolithic resulted in
75 demographic explosion and increasing human pressure on the environment (Gignoux *et al.*,
76 2011, Shennan *et al.*, 2013), leading to progressive deforestation of the continent (Kaplan *et*
77 *al.*, 2009, Roberts *et al.*, 2018). The transformation of Europe's landscapes from a nature-
78 dominated to a more human-dominated state has been long and complex (Fyfe *et al.* 2015,
79 Roberts *et al.* 2018). It created dynamic habitat conditions and strongly limited access to
80 preferred habitats for wild animals, and influenced their distribution, densities, fitness or food
81 habits, and in the worst cases led to population extirpations or species extinctions (Crees *et*
82 *al.*, 2016, Pavelková Řičánková *et al.*, 2015, Rosvold *et al.*, 2013).

83 The majority of the abundant Pleistocene megafauna in Europe became extinct before
84 the start of the Holocene (Elias & Schreve, 2007, Pacher & Stuart, 2009, Stuart, 1991). The
85 major patterns of postglacial changes in Palearctic mammalian diversity were not extinctions
86 but rather radical shifts of species distribution ranges and retreat northwards or eastwards
87 (Pavelková Řičánková *et al.*, 2015). Few species of large European mammals survived the

88 Pleistocene/Holocene transition, and inhabited the European continent during the subsequent
89 millennia (Benecke, 2005, Pavelková Řičánková *et al.*, 2015, Schmölcke & Zachos, 2005).
90 These species were exposed to several processes related to climate and habitat change, as well
91 as increasing human pressure (Hofman-Kamińska *et al.*, 2018b, Rosvold *et al.*, 2013). Two
92 species, European bison (*Bison bonasus*) and moose (*Alces alces*), survived until present
93 (moose) or were restored in the wild from captive survivors after extirpation at the beginning
94 of the 20th century (European bison). Aurochs (*Bos primigenius*), widely distributed in Europe
95 during the Pleistocene, survived into the Holocene but finally became extinct during the early
96 17th century (Van Vuure, 2005).

97 The feeding habits of these large herbivores have been detected by stable isotope
98 analysis in the Pleistocene/Holocene transition and at the beginning of the Holocene
99 (12,000±600 - 10,022±229 cal yrs BP) (Bocherens *et al.*, 2015). These feeding habits ranged
100 from grazing in aurochs, to mixed-feeding in European bison, and to browsing in moose,
101 reflecting feeding types determined by digestive system classification (Hofmann, 1989) and
102 probably represent the natural feeding behaviour of these species. Changes in the diet patterns
103 of these large herbivores have been identified by dental microwear textural analysis (DMTA)
104 (Hofman-Kamińska *et al.*, 2018b) and may indicate behavioural changes in response to
105 environmental changes, as well as different periods of favorable conditions during the
106 Holocene in Europe. Forested areas, favorable for browsers such as moose, but marginal for
107 grazers such as aurochs and European bison, might have created refugia from increasing
108 human pressure during the mid-Holocene and promoted the survival of these latter species in
109 forest habitats, but in circumstances of lowered fitness and population densities. This may
110 have led to increased stochasticity and population fragmentations, and local extinctions
111 (Cromsigt *et al.*, 2012, Kerley *et al.*, 2012). Opening of forest habitats would have created
112 more optimal foraging conditions for grazers, less for browsers, but might have exposed them

113 to increased human pressure and had similar effects (reduced fitness, population
114 fragmentation and local extinction). Large mammals are particularly vulnerable to
115 environmental changes and habitat fragmentations and exposed to risk of extirpation or
116 extinction due to their body size, lower densities, larger spatial requirements, and
117 disproportional exploitation by humans, particularly more specialized species that have a
118 lower capacity for adaptation (Cardillo *et al.*, 2005, Keinath *et al.*, 2017). Did they adapt to
119 the limits set by their morphological characteristics and foraging plasticity or not? What
120 processes promoted their survival or led to their disappearance as habitat structure changed?
121 Reconstruction of foraging habitats and dietary behavior may shed light on how large
122 herbivores coped with changing environmental conditions.

123 Stable carbon and nitrogen isotopic ratios are increasingly used as environmental
124 and dietary indicators for modern as well as for fossil herbivores (Bocherens *et al.*, 2009,
125 Bocherens *et al.*, 2015, Britton *et al.*, 2012, Drucker *et al.*, 2003, Drucker *et al.*, 2010, García
126 *et al.*, 2009, Gąsiorowski *et al.*, 2014, Gron & Rowley-Conwy, 2017, Hofman-Kamińska *et*
127 *al.*, 2018a, Jürgensen *et al.*, 2017, Noe-Nygaard *et al.*, 2005, Šturm *et al.*, 2017). In this study
128 we used stable carbon and nitrogen isotope compositions from radiocarbon dated bone
129 samples as proxies for foraging habitats and diet of European bison, moose and aurochs
130 throughout the Holocene in Europe. By studying the foraging ecology of three European large
131 herbivore species over millennial timescales during the Holocene we aimed to: (1) identify
132 habitat preferences and diets before and after the Neolithic environmental transformations; (2)
133 analyze the response of the largest remaining Pleistocene megafauna in Europe to the major
134 environmental changes of the Holocene (from increasing tree cover during the Early and pre-
135 Neolithic Holocene to increasing habitat openness during the Neolithic and subsequent time
136 periods); and (3) identify factors influencing their patterns of habitat use and diet. We
137 hypothesized that forest expansion in the early Holocene forced large herbivores, especially

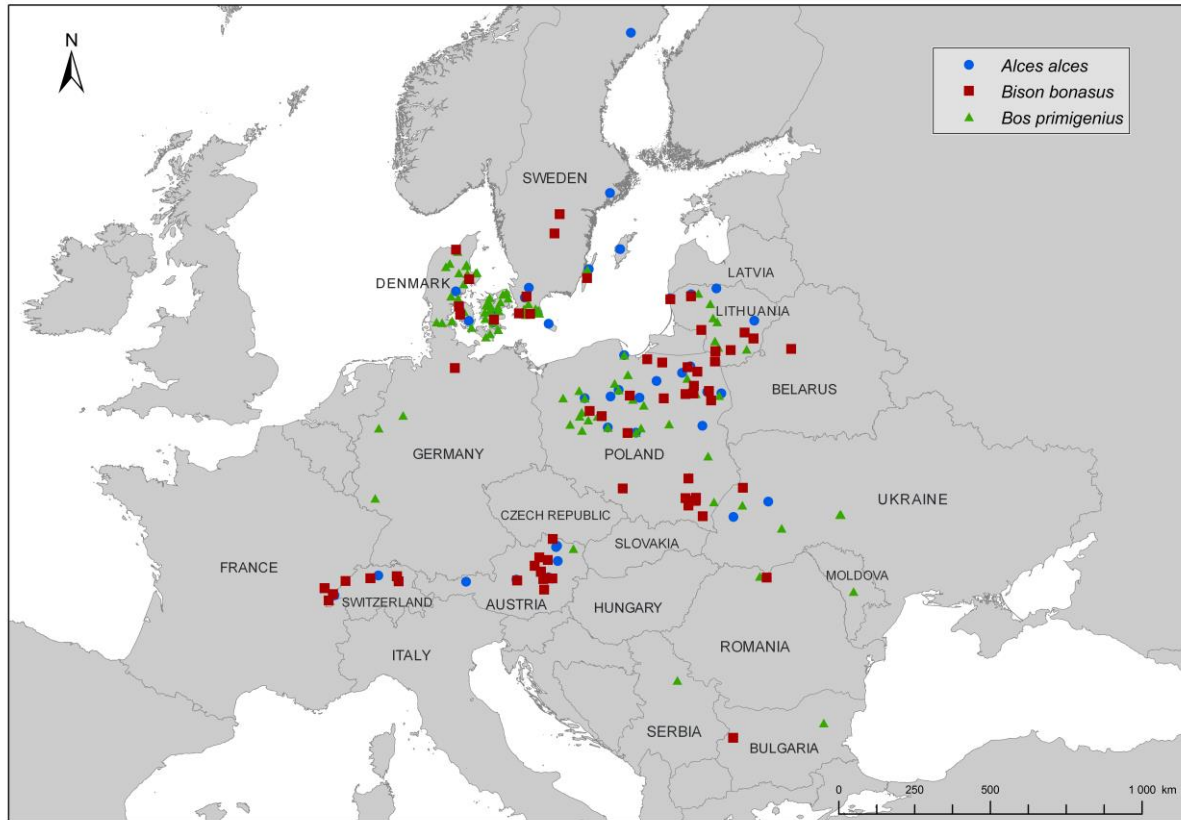
138 those primarily adapted to grazing (aurochs and European bison) into forests as refugial
139 habitats, to shift their pattern of habitat use, resulting in stable isotopic signatures change.
140 Subsequently, increasing human pressure did not allow large herbivores to return to more
141 open habitats, despite the creation of open habitats resulting from the deforestation of
142 Europe in the Late Holocene.

143

144 **MATERIALS AND METHODS**

145 **Sample collection and age determination**

146 A total of 295 bone samples, including 121 European bison, 91 aurochs and 83 moose
147 originating from 14 European countries were collected from paleontological, zoological and
148 private collections (Table S1) in Central and Eastern Europe (Figure 1). We validated the
149 species identification (especially for European bison and aurochs bones) (Gee, 1993), by
150 comparing characteristic morphological features using modern European bison and moose
151 bones from zoological collection of the Mammal Research Institute PAS in Białowieża and
152 other well identified historical specimens as reference material. Additionally, we included into
153 the analysis published stable carbon isotopic data for 69 specimens (19 moose, 5 European
154 bison and 45 aurochs) and stable nitrogen isotopic data for 36 specimens (12 moose, 5
155 European bison and 19 aurochs) available in the literature (Antanaitis-Jacobs *et al.*, 2009,
156 Bocherens *et al.*, 2015, Fornander *et al.*, 2008, Gravlund *et al.*, 2012, Jessen *et al.*, 2015,
157 Lidén *et al.*, 2004, Linderholm *et al.*, 2014, Noe-Nygaard *et al.*, 2005). In total, 364 large
158 herbivore bone samples and literature records were used in the study (Figure 1).



159

160

161 Figure 1. Distribution of localities with specimens of moose (*Alces alces*) (blue circles),
 162 European bison (*Bison bonasus*) (red squares) and aurochs (*Bos primigenius*) (green triangles)
 163 in the Holocene.

164

165 Age determinations of the bone samples were based on radiocarbon dating from the
 166 literature or museum data, or derived from archeological context. For 94 specimens without
 167 any, or uncertain, age determination we performed direct radiocarbon dating at the Laboratory
 168 of Ion Beam Physics, Eidgenössische Technische Hochschule Zürich, Switzerland (Swiss
 169 Federal Institute of Technology Zürich) (ETH). All radiocarbon dates were calibrated to BP
 170 dates with 1σ (95.4%) probability using the IntCal13 calibration curve in OxCal v4.2 (Reimer
 171 *et al.*, 2013). In total 186 bone samples were ^{14}C dated, another 178 had a well identified

172 archeological context, in most of cases confirmed by additional radiocarbon dates performed
173 for this study.

174 This study is unique in incorporating the analysis of an unprecedented number of
175 European bison (*Bison bonasus*) bone material (126), (distributed widely across the European
176 continent), a species with a very poorly studied Holocene history. Additionally, we provide a
177 very large number of new ¹⁴C radiocarbon dated bones for this species (59). Before this study
178 only 32 radiocarbon dated European bison specimens were published (Blant & Wenger, 2010,
179 Bocherens *et al.*, 2015, Ekström, 1993, Herrmann *et al.*, 2010, Hofman-Kamińska *et al.*,
180 2018b, Soubrier *et al.*, 2016, Spitzenberger, 2002, Węcek *et al.*, 2016).

181

182 **Collagen extraction and stable isotope analysis**

183 Small pieces of compact bone (0.7 g) were cleaned with acetone and distilled water in
184 an ultrasound bath in order to remove dust and potential glue remains, before being crushed to
185 a powder and sieved to obtain a grain size no larger than 0.7 mm. Collagen for the isotopic
186 measurements was prepared according to the protocol described by Bocherens *et al.*
187 (Bocherens *et al.*, 1997).

188 The elemental and isotopic measurements were performed at the Department of
189 Geosciences at the University of Tübingen (Germany) using an elemental analyzer NC 2500
190 connected to a Thermo Quest Delta+XL mass spectrometer. The isotopic ratios were
191 expressed using the “δ” (delta) value as follows: $\delta^{13}\text{C} = [({}^{13}\text{C}/{}^{12}\text{C})_{\text{sample}} / ({}^{13}\text{C}/{}^{12}\text{C})_{\text{reference}} - 1] \times$
192 $1000(\text{‰})$, $\delta^{15}\text{N} = [({}^{15}\text{N}/{}^{14}\text{N})_{\text{sample}} / ({}^{15}\text{N}/{}^{14}\text{N})_{\text{reference}} - 1] \times 1000(\text{‰})$. The internationally defined
193 standards were V-PDB for $\delta^{13}\text{C}$ values and atmospheric nitrogen (AIR) for $\delta^{15}\text{N}$ values.
194 Samples of collagen were normalized to $\delta^{13}\text{C}$ values of USGS24 ($\delta^{13}\text{C} = -16.00\text{‰}$) and to $\delta^{15}\text{N}$
195 values of IAEA 305A ($\delta^{15}\text{N} = 39.80\text{‰}$). The reproducibility was $\pm 0.1\text{‰}$ for $\delta^{13}\text{C}$ and $\pm 0.2\text{‰}$

196 for $\delta^{15}\text{N}$ measurements based on multiple analysis of purified collagen from modern bones (n
197 > 175).

198 The reliability of the isotopic signatures of the collagen extracts was addressed using
199 their chemical composition. Only high quality extracts with %C, %N, and C/N similar to those
200 of collagen extracted from fresh bone were used for isotopic measurements. Therefore, only
201 collagen extracts that had atomic C/N ratios with $2.9 \leq \text{C/N} \leq 3.6$ (DeNiro, 1985), %C > 8%
202 and %N > 3% were taken for the analysis (Ambrose, 1990).

203

204 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ variables

205 In European temperate and boreal ecosystems, woody and herbaceous plants follow the C_3
206 photosynthetic pathway and have $\delta^{13}\text{C}$ values ranging from -35‰ to -20‰ (Dawson *et al.*,
207 2002). The fraction of the vegetation that is C_4 is less than 0.1 and concentrates mostly in
208 southern Europe (Still *et al.*, 2003). Plants growing under the canopy of densely forested
209 environments exhibit lower $\delta^{13}\text{C}$ values in comparison to plants from open conditions
210 (Bonafini *et al.*, 2013, Broadmeadow *et al.*, 1992, Gebauer & Schulze, 1991, Van der Merwe
211 & Medina, 1991). Such a depletion in $\delta^{13}\text{C}$ is reflected in the bone collagen of forest-dwelling
212 herbivores in boreal and temperate forests (Drucker & Bocherens, 2009, Drucker *et al.*, 2008),
213 with a tentative threshold $\delta^{13}\text{C}$ value for foraging under dense canopy forest at -22‰,
214 meaning that herbivores with such collagen $\delta^{13}\text{C}$ values have been foraging essentially under
215 a dense canopy cover (Drucker *et al.*, 2008). Recent studies conducted on modern European
216 bison and moose confirm that with increasing percentage of forest cover, stable carbon
217 isotope concentration in collagen of large herbivores decreases (Hofman-Kamińska *et al.*,
218 2018a). It confirms also that stable carbon isotopes in herbivore collagen, which is
219 continuously replaced through the whole animal's life by the incorporation of new atoms of
220 carbon deriving from the diet, reflects foraging in densely forested *versus* open landscape

221 conditions. Such patterns allow us to reconstruct the foraging habitats of large herbivores in
222 the past on the basis of $\delta^{13}\text{C}$ values.

223 $\delta^{15}\text{N}$ values differ between plant types. Non-mycorrhizal plants such as graminoids,
224 forbs and clubmosses exhibit higher $\delta^{15}\text{N}$ values than ectomycorrhizal and ericoid plants, such
225 as trees and shrubs (Ben-David *et al.*, 2001, Craine *et al.*, 2009, Emmerton *et al.*, 2001,
226 Hobbie *et al.*, 2005, Kristensen *et al.*, 2011, Schulze *et al.*, 1994). Intermediate values of $\delta^{15}\text{N}$
227 are found in mosses (Craine *et al.*, 2009, McLeman, 2006, Michelsen *et al.*, 1998, Michelsen
228 *et al.*, 1996). These results allow us to reconstruct diet type based on stable nitrogen isotope
229 compositions, even if it is not possible to provide a threshold $\delta^{15}\text{N}$ value for bone collagen of
230 grazers versus browsers due to the impact of local environmental factors, but in a given
231 context, grazers typically exhibit higher $\delta^{15}\text{N}$ values than browsers (Bocherens, 2003,
232 Bocherens, 2015). It has been shown that the $\delta^{15}\text{N}$ values in plants decline with increasing
233 elevation (Huber *et al.*, 2007, Sah & Brumme, 2003, Sparks & Ehleringer, 1997). This is
234 probably connected with lower mineralization and lower net nitrification rates induced by
235 more abundant rainfall and lower temperatures at higher elevation (Liu & Wang, 2010).

236

237 **Forest cover and spatial data**

238 We extracted total tree cover (hereafter described as forest cover), deciduous tree cover and
239 needle-leaf tree cover , values for each bone specimen from the published dataset of (Fyfe *et*
240 *al.*, 2015) using the geographic coordinates of herbivore bones, and their calibrated age. This
241 generated data that described that nature of vegetation for the location and time period of
242 every sample. The estimates of forest cover in Fyfe *et al.* (2015) were generated using data
243 from the European Pollen Database (Fyfe *et al.*, 2009, Leydet, 2007–2018). Pollen data from
244 individual site records were aggregated into contiguous 200-year long time windows between
245 18,000 and 0 yrs BP using the chronologies in (Giesecke *et al.*, 2014) and transformed from

246 pollen proportions to % land cover classes (LCCs) using the pseudobiomization approach
247 (Fyfe *et al.*, 2010). The resulting values were interpolated to produce the spatially-continuous
248 estimates of forest cover at 20 km resolution for each time window using a thin-plate spline
249 with elevation as a co-variate (Fyfe *et al.*, 2015).

250 The elevation for each specimen was extracted from the global raster data grids from
251 the Global Multi-resolution Terrain Elevation Data 2010 (GMTED2010) in ArcGIS 10.5.0
252 using spatial analysis tools (ESRI, 2017). We used median 7.5 arc-seconds resolution, which
253 has a root mean square error (RMSE) range between 29 and 32 meters (Danielson & Gesch,
254 2011).

255

256 **Statistical analysis**

257 In order to obtain the age of radiocarbon dated samples for statistical analyzes, we calculated
258 an average between the lower and higher calibrated age range. We applied the same procedure
259 to determine the average age for specimens dated archaeologically, e. g. for a specimen dated
260 between 16-17th century, we assumed the age of 400 cal years BP. First, we tested differences
261 in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between moose, European bison and aurochs for the whole of the Holocene
262 with ANOVA. The normality of the variables' distribution was tested with Shapiro-Wilk test.
263 To check homogeneity of variance assumption, Brown-Forsythe and Levene tests were
264 performed for each period. When ANOVAs showed statistically significant differences, post
265 hoc comparisons were performed using Tukey's HSD test for unequal N. To test whether
266 Neolithic agriculture impacted $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of large herbivores, we divided the Holocene
267 specimens according to the criteria of Neolithization (the timing of the Neolithic varies across
268 our sites in Europe but is broadly between 7000 and 2600 cal yrs BP). The Early and pre-
269 Neolithic Holocene specimens included those from regions where Mesolithic cultures
270 (between 12,000 and 6000 cal yrs BP) still occurred according to regional studies and

271 Neolithic and Late Holocene specimens from locations in space and time where Neolithic and
272 later agriculture was established (Antanaitis-Jacobs *et al.*, 2009, Deak *et al.*, 2018, Puhe &
273 Ulrich, 2001). We explored changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ variables between the two periods for
274 each of the species separately. In the next step we performed one-way ANOVA to study
275 differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between European bison, moose and aurochs in each of the two
276 periods.

277 We ran separate models for each of the two stable isotopes for each of the three
278 species to investigate factors influencing $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in bone collagen (in total 6 models).
279 Due to the high correlation ($R > 0.5$), from the whole set of available explanatory variables:
280 percentage of the total forest cover, percentage of deciduous forest, percentage of needle-leaf
281 forest, age, longitude, latitude and elevation we excluded percentage of deciduous forest
282 (highly correlated with percentage of the total forest cover; R from 0.74 to 0.81 in different
283 species) and latitude (highly correlated with elevation; R from 0.75 to 0.81 in different
284 species). Percentage forest cover was not available for all our specimens, due to the lack of
285 pollen data for the oldest specimens, or absence of precise dating of faunal material, therefore
286 for modeling we used only records with complete datasets (among 69 records from the
287 literature we used only 22 complete datasets). Due to the large discrepancy of scale ranges (6-
288 29° in longitude vs. 0-12000 cal yrs BP in time), we standardized our explanatory variables in
289 R to have a mean of 0 and a sd of 0.5 using function `standardize.y` (Gelman & Hill, 2007). We
290 then ran multiple linear regression models with single isotope abundance as the response
291 variable. The Akaike Information Criterion (AIC) with the second-order correction for a small
292 sample size (AICc) was used for model ranking. We did not find a singular best model within
293 any of the models run, so we applied model averaging where cumulative weights of subsets of
294 models did not exceed 0.95. We looked at full model averaging to identify factors
295 significantly affecting $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ variables. The normality and homoscedasticity in the

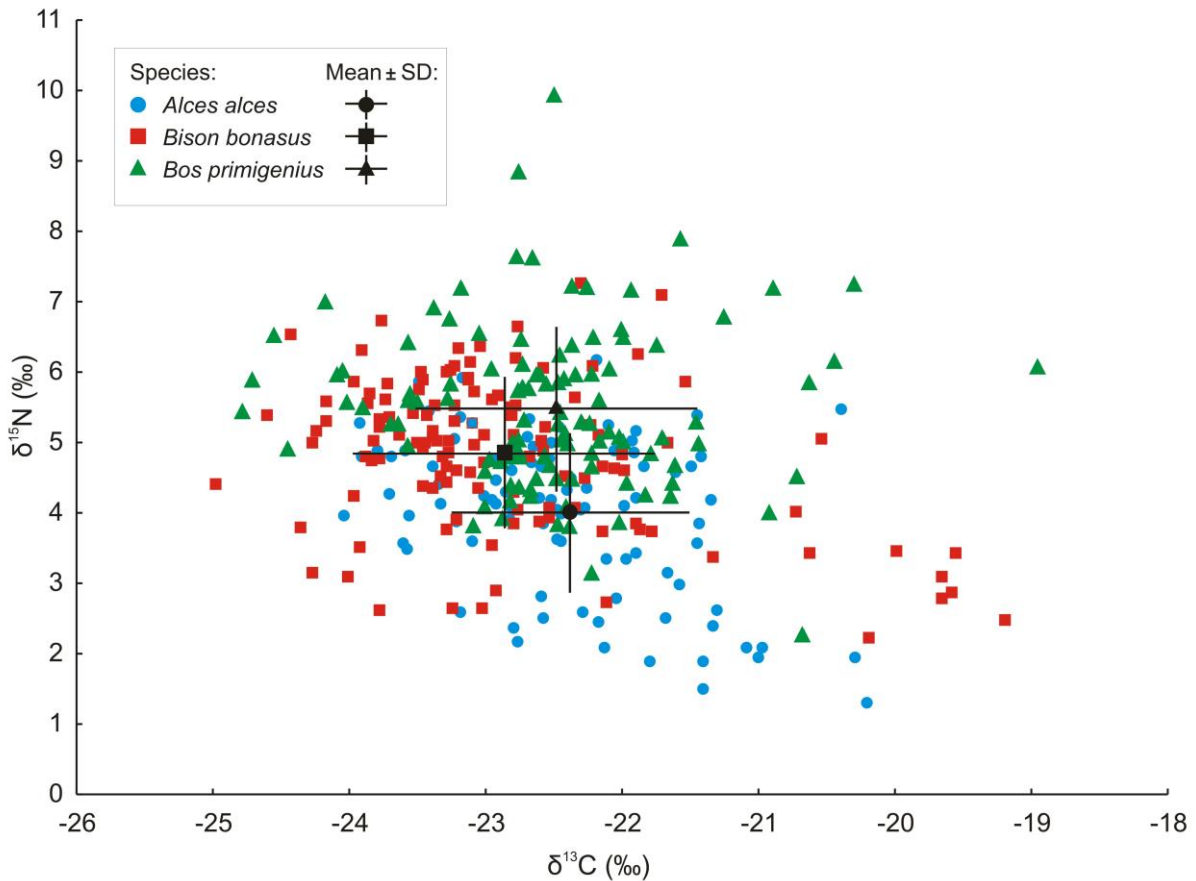
296 distribution of final model residuals was tested by inspection of the quantile–quantile
297 distribution plot and model residuals against fitted values (estimated responses) plot. We
298 checked all models for spatial autocorrelation. Semi-variograms showed no spatial
299 autocorrelation in model residuals (Zuur *et al.*, 2009). Multiple regression models were
300 completed in R (version 3.4.4) (R-Core-Team, 2016). Model ranking was done using the
301 package MuMIn (Bartoń, 2015). All ANOVAs were performed in Statistica (version 9.1)
302 (StatSoft, 2010).

303

304 **RESULTS**

305 **Stable isotope signatures of large herbivores during the Holocene**

306 At the scale of the whole Holocene, the three species of large herbivores differ in their $\delta^{13}\text{C}$
307 values ($F = 5.43$, $p = 0.005$, $N = 364$). Aurochs ($-22.5 \pm 1.0\%$) and moose ($-22.4 \pm 0.9\%$) have
308 significantly higher mean $\delta^{13}\text{C}$ values than European bison ($-22.8 \pm 1.1\%$) ($p = 0.04$ and $p =$
309 0.01 , respectively). All three species significantly differ in their $\delta^{15}\text{N}$ values ($F = 45.04$, $p <$
310 0.0001 , $N = 332$). Moose are characterized by the lowest ($+4.0 \pm 1.1\%$), European bison by
311 intermediate ($+4.9 \pm 1.1\%$) and aurochs by the highest ($+5.5 \pm 1.2\%$) $\delta^{15}\text{N}$ values ($p < 0.0001$, p
312 < 0.0001 and $p < 0.0001$, respectively) (Figure 2).



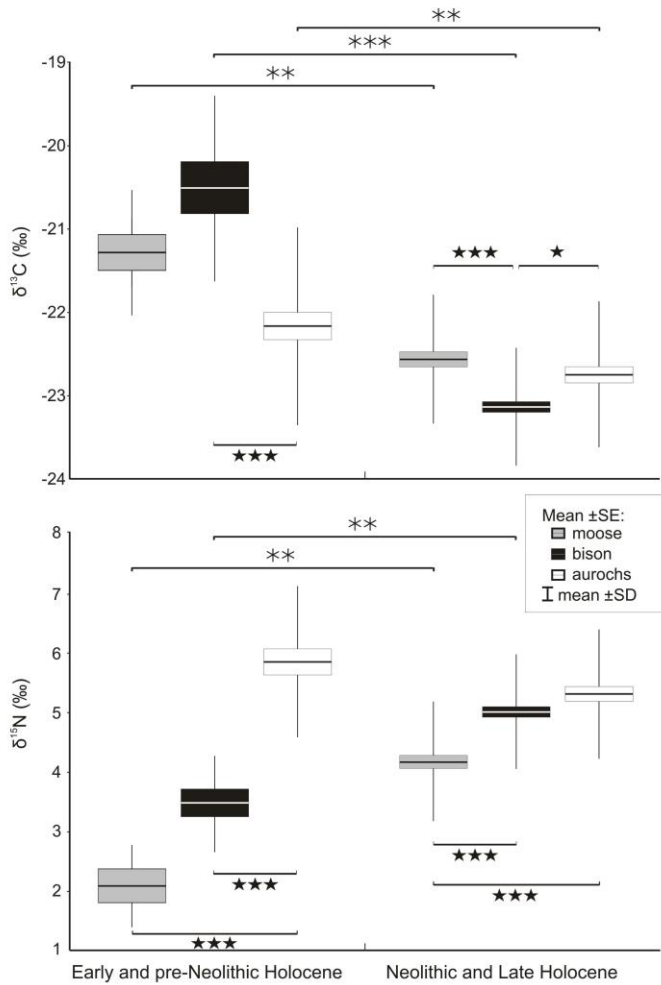
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315 Figure 2. Stable carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope signatures of moose (*Alces alces*)
 316 (blue circles), European bison (*Bison bonasus*) (red squares) and aurochs (*Bos primigenius*)
 317 (green triangles) with mean values and standard deviations.

318

319 We find that moose, European bison and aurochs samples have significantly higher
 320 mean $\delta^{13}\text{C}$ values in the Early and pre-Neolithic Holocene in comparison to the Neolithic and
 321 Late Holocene ($p = 0.0002$, $N = 97$; $p < 0.0001$, $N = 126$; $p = 0.003$, $N = 126$; respectively)
 322 (Figure 3). European bison and moose have significantly higher mean $\delta^{15}\text{N}$ values in the
 323 Neolithic and Late Holocene ($p = 0.0006$, $N = 97$; $p < 0.0001$, $N = 126$; respectively)
 324 compared to the Early and pre-Neolithic Holocene. Aurochs do not differ in mean $\delta^{15}\text{N}$
 325 between those two periods ($p = 0.06$, $N = 100$) (Figure 3).



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Figure 3. Differences in stable carbon $\delta^{13}\text{C}$ and nitrogen $\delta^{15}\text{N}$ isotope signatures between moose, European bison and aurochs in the Early and pre-Neolithic and in the Neolithic and Late Holocene sample groups and changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between the two periods. Asterisks indicate significant differences for each species between the two periods ** - for $p = 0.01$, ***- for $p < 0.001$, (pairwise differences for Tukey's HSD test for unequal N) and significant changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between three species in each of the period *- for $p = 0.01$, ***- for $p < 0.0001$.

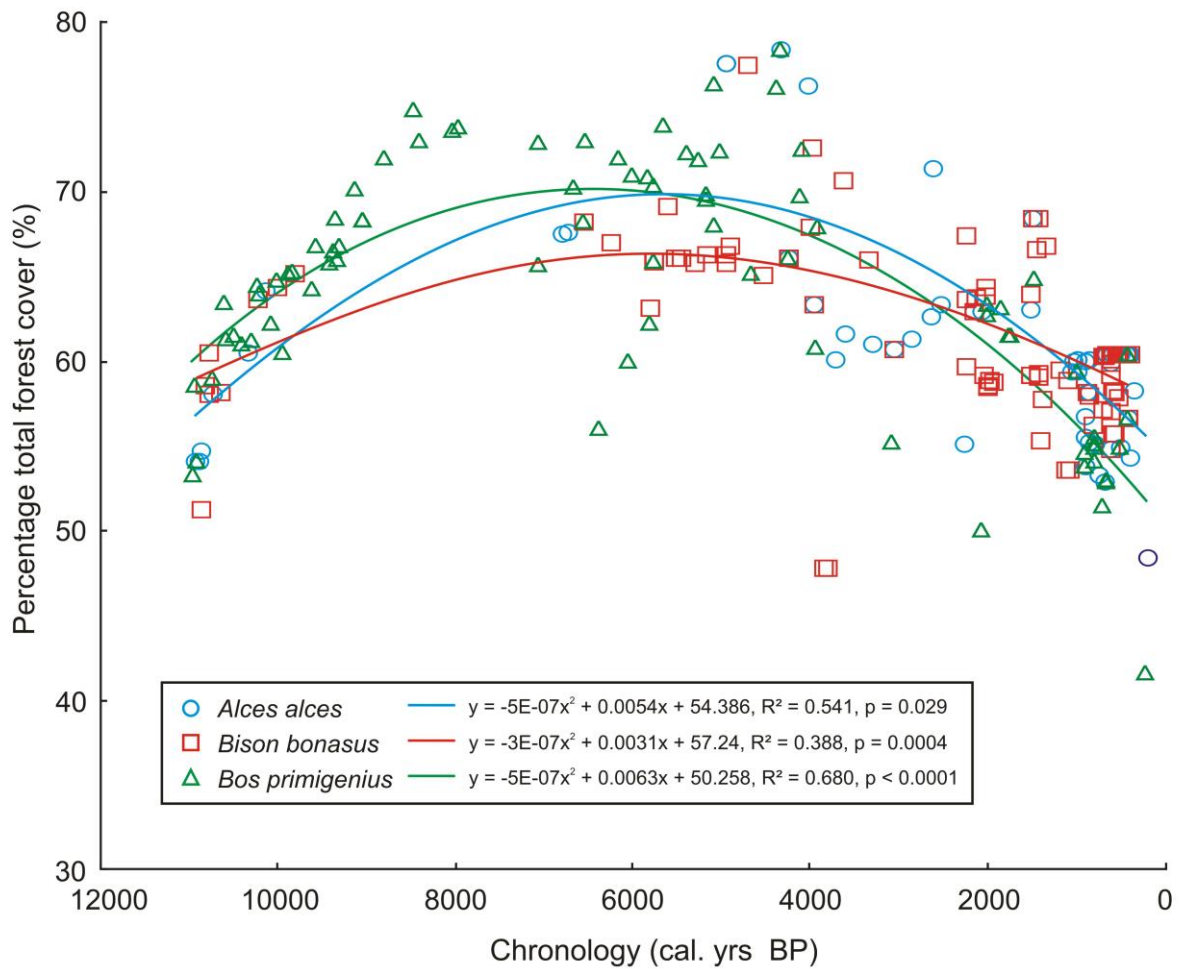
The analysis of variance we use to explore differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between European bison, moose and aurochs shows significant differences in both $\delta^{13}\text{C}$ ($F = 12.70$, $p < 0.0001$, $N = 79$; $F = 14.10$, $p < 0.0001$, $N = 270$, in the Early and pre-Neolithic and the

338 Neolithic and Late Holocene respectively) and in $\delta^{15}\text{N}$ values ($F = 42.50$, $p < 0.0001$, $N = 53$;
339 $F = 28.86$, $p < 0.0001$, $N = 263$, in the Early and pre-Neolithic Holocene and the Neolithic and
340 Late Holocene respectively) in each of the period (Figure 3). During the Early and pre-
341 Neolithic Holocene significantly lower mean $\delta^{13}\text{C}$ values are found in aurochs ($-22.2 \pm 1.2\text{‰}$)
342 than in European bison ($-20.6 \pm 1.1\text{‰}$,) ($p = 0.001$) (Figure 3). Moose has an intermediate
343 mean $\delta^{13}\text{C}$ value ($-21.3 \pm 0.8\text{‰}$). In the Neolithic and Late Holocene, the mean $\delta^{13}\text{C}$ value
344 recorded in European bison ($-23.1 \pm 0.7\text{‰}$), is significantly lower than in moose ($-22.6 \pm 0.8\text{‰}$,
345 $p < 0.0001$) and aurochs ($-22.8 \pm 0.9\text{‰}$, $p = 0.007$). In the Early and pre-Neolithic Holocene,
346 the highest mean $\delta^{15}\text{N}$ value registered in aurochs ($+5.8 \pm 1.2\text{‰}$) is significantly different than
347 in European bison ($+3.5 \pm 0.9\text{‰}$) ($p = 0.0001$) and moose ($+2.1 \pm 0.7\text{‰}$) ($p = 0.0001$) (Figure 3).
348 In the Neolithic and Late Holocene, the lowest mean $\delta^{15}\text{N}$ registered in moose ($+4.2 \pm 1.0\text{‰}$)
349 significantly differs from those in European bison ($+5.0 \pm 1.0\text{‰}$) ($p < 0.0001$) and aurochs
350 ($+5.3 \pm 1.1\text{‰}$) ($p < 0.0001$) (Figure 3).

351

352 **Factors influencing $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in large herbivores**

353 The percentage total forest cover for moose, European bison and aurochs locations shows a
354 similar temporal pattern with increase in the Early Holocene until the start of the Neolithic and
355 then decreasing throughout the Late Holocene (Figure 4).



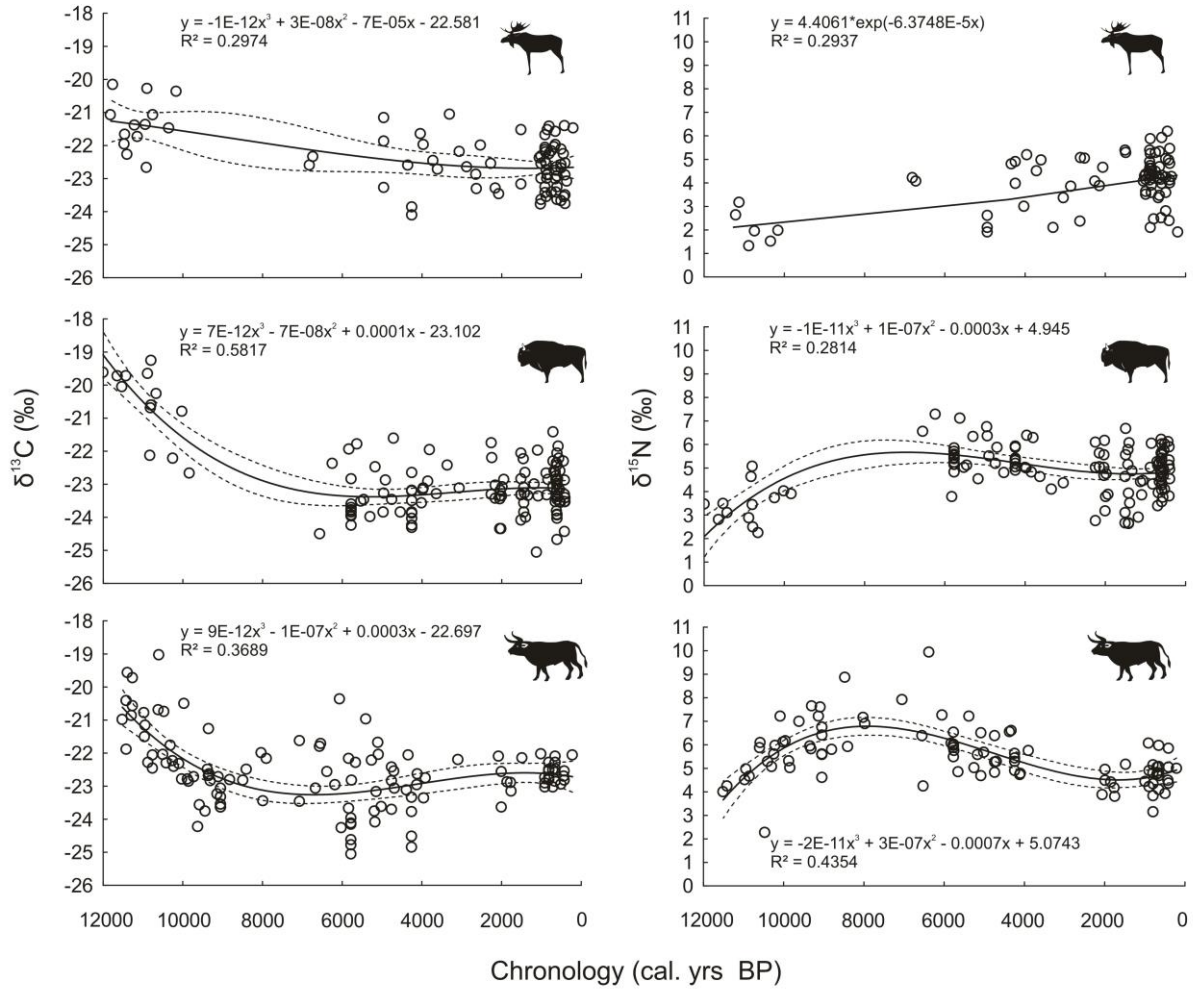
356

357 Figure 4. Percentage total forest cover in the Holocene for moose (*Alces alces*) (circles),
 358 European bison (*Bison bonasus*) (squares) and aurochs (*Bos primigenius*) (triangles) bone
 359 locations.

360

361 The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in bones of large herbivores show some fluctuations
 362 throughout the Holocene with the pattern differing between the three species (Figure 5).

363



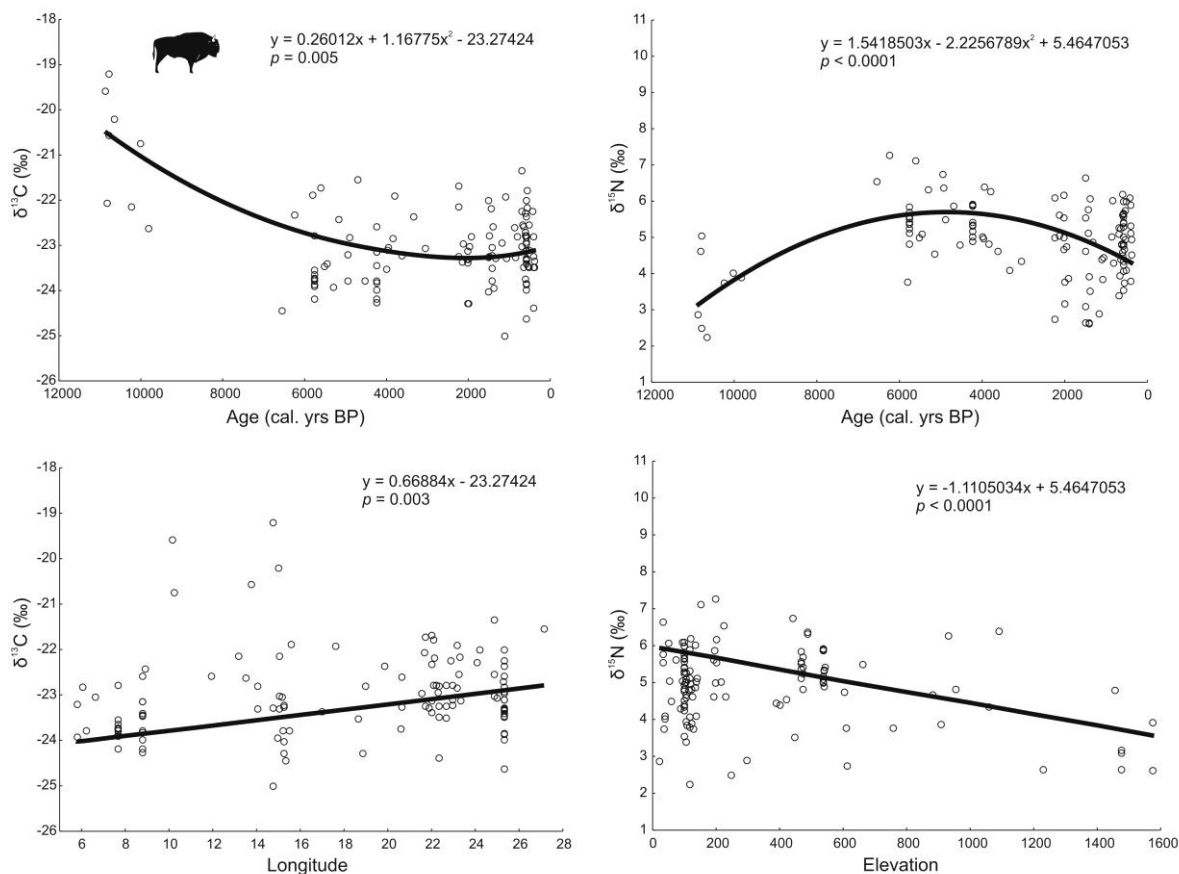
364

365 Figure 5. Chronological changes in stable carbon $\delta^{13}\text{C}$ and nitrogen $\delta^{15}\text{N}$ isotope signatures of
 366 moose, European bison and aurochs during the Holocene. The solid lines represent estimated
 367 regression lines and the dotted lines 0.95 confidence intervals.

368

369 The average model shows that none of the analysed factors significantly affected $\delta^{13}\text{C}$
 370 and $\delta^{15}\text{N}$ stable isotope abundances in moose (Table S2, Table S3). For European bison, the
 371 ranges of R^2 of competing models used in averaging were between 0.3988 - 0.4235 and the
 372 average model shows significant effect of age and longitude on $\delta^{13}\text{C}$ stable carbon isotope
 373 signatures (Table S2, Table S3). The $\delta^{13}\text{C}$ values decrease in time in the first half of the
 374 Holocene, then stabilize during the following period, and increase with longitude (Table S3,
 375 Figure 6). The ranges of R^2 of competing models used in averaging were between 0.3720 -

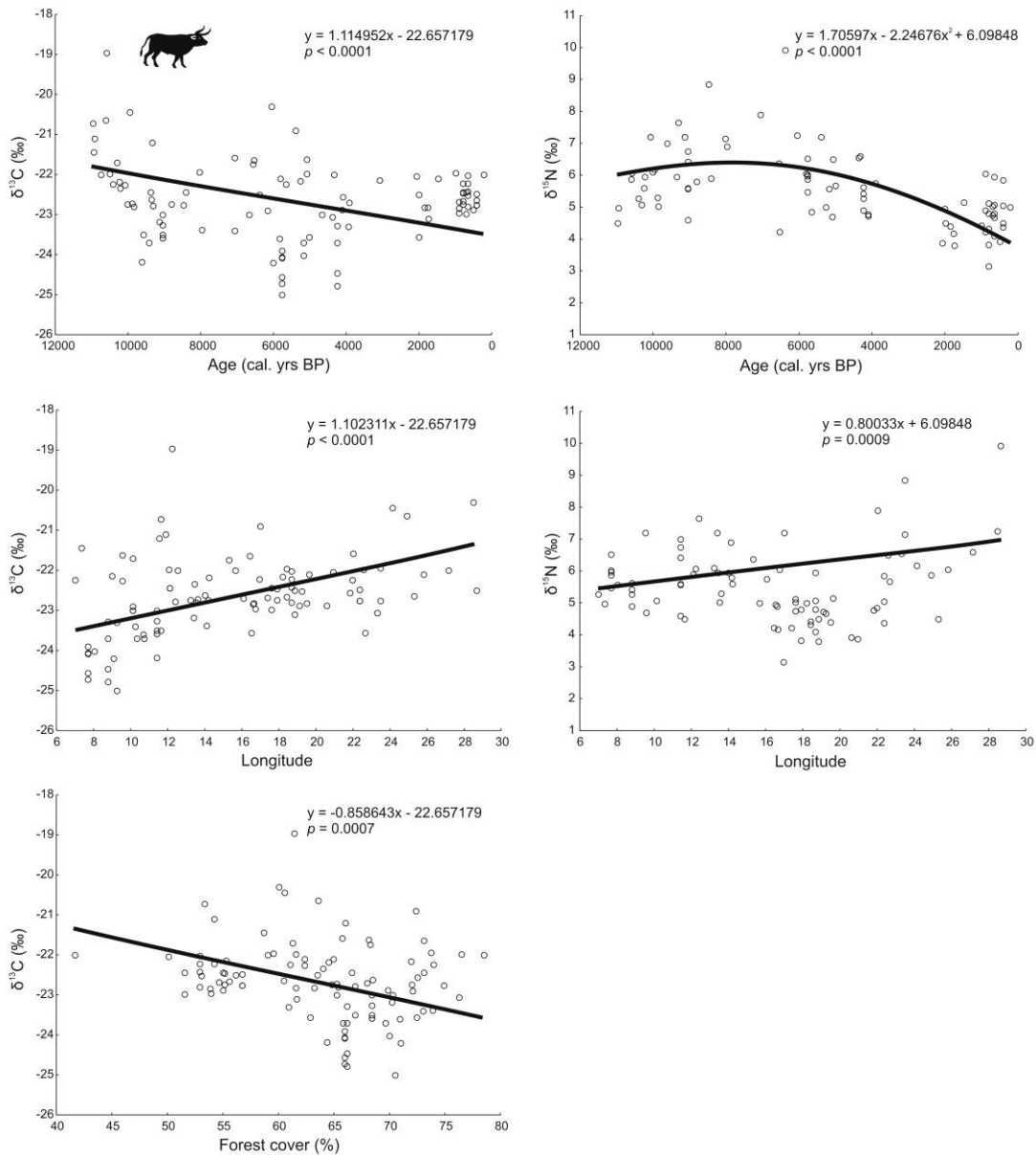
376 0.3793 and the average model indicates that $\delta^{15}\text{N}$ values in European bison are significantly
 377 affected by age and elevation (Table S2, Table S3). The $\delta^{15}\text{N}$ concentration increases through
 378 time in the first half of the Holocene, then slightly decreases (Figure 6). With increasing
 379 elevation, $\delta^{15}\text{N}$ values significantly decrease (Figure 6).



380
 381 Figure 6. Influence of the age, longitude and elevation on stable carbon ($\delta^{13}\text{C}$) and nitrogen
 382 ($\delta^{15}\text{N}$) isotope signatures of the European bison (*Bison bonasus*) in the Holocene.

383
 384 The ranges of R^2 of competing models used in averaging were between 0.4273 - 0.4443
 385 and the average model for aurochs shows significant effect of age, longitude and total forest
 386 cover on $\delta^{13}\text{C}$ values (Table S2, Table S3). The $\delta^{13}\text{C}$ values decrease through time and with
 387 increasing forest cover, and increase with increasing longitude, i.e. from West to East (Table
 388 S3, Figure 7). The ranges of R^2 of competing models used in averaging were between 0.4025 -

389 0.4174 and the average model shows a significant effect of age and longitude on $\delta^{15}\text{N}$ values
 390 in aurochs (Table S2, Table S3). The $\delta^{15}\text{N}$ values decrease in time, while increasing with
 391 longitude, therefore becoming higher from West to East (Figure 7).



392

393 Figure 7. Influence of the age, longitude and forest cover on stable carbon ($\delta^{13}\text{C}$) and nitrogen
 394 ($\delta^{15}\text{N}$) isotope signatures of aurochs (*Bos primigenius*) in the Holocene.

395

396 DISCUSSION

397 Stable isotope composition of large herbivores during the Holocene

398 Reconstruction of the habitat use and diet of large herbivores inhabiting Central and Eastern
399 Europe has shown a wide variation of foraging habitats and diets of moose, European bison
400 and aurochs throughout the Holocene. Comparing mean values of isotopic signatures in the
401 scale of the whole Holocene, we found that moose and aurochs more often occupied open
402 habitats and less forested areas, reflected in their higher mean $\delta^{13}\text{C}$ values, while European
403 bison more often foraged in forested habitats. Differences among the diets of the three species
404 are reflected in their $\delta^{15}\text{N}$ values and predict different foraging niches with moose being the
405 most browsing, and aurochs the most grazing species (Hofmann, 1989).

406 The selection of early successional forests and tundra subalpine areas by moose before
407 the forest maximum and later stream valley shrub habitats and forest gaps, which offer high
408 availability of browse and cover, reflects the historical selection of relatively open habitats and
409 woody diet of this large herbivore (Czernik *et al.*, 2013, Morow, 1976, Olsson *et al.*, 2011,
410 Wam & Hjeljord, 2010b). Whilst the distribution range of moose strongly contracted eastward,
411 the species did not experience extirpation in the wild and is now recolonizing its historical
412 range (Niedziałkowska *et al.*, 2016a, Niedziałkowska *et al.*, 2016b, Schmölcke & Zachos,
413 2005).

414 According to $\delta^{15}\text{N}$ values, aurochs in the Holocene had more herbaceous plants in the
415 diet than two other species. This is consistent with the previous stable isotope nitrogen
416 analysis of bone collagen from Scandinavia and England confirming grazing of the aurochs,
417 which is similar to cattle foraging behaviour (Lynch *et al.*, 2008, Noe-Nygaard *et al.*, 2005).
418 Intermediate between the most grazing aurochs and the most browsing moose nitrogen isotope
419 composition of European bison supports earlier findings on its mixed diet and plasticity in
420 adaptation to utilize a wide range of forest habitats (Bocherens *et al.*, 2015, Hofman-Kamińska
421 *et al.*, 2018a, Hofman-Kamińska *et al.*, 2018b, Kowalczyk *et al.*, 2011, Merceron *et al.*, 2014).
422 This flexibility may result from the European bison's evolutionary adaptations to various food

423 resources and habitats as reflected by genome analysis and gene selection (Gautier *et al.*,
424 2016).

425 Our data show that following deglaciation of northern Europe, in the earliest phase of
426 the Holocene (between 11.6 - 10.5 ka cal yrs BP) moose, European bison and aurochs from
427 Scandinavia and northern Europe occupied relatively more open habitats, which at that time
428 were open tundra and shrubland with undeveloped forest (Björck *et al.*, 2002, Jessen *et al.*,
429 2015). Later, before the advent of the Neolithic, between 9.5 ka cal yrs BP and 7 ka cal yrs BP,
430 aurochs stayed in more forested habitats, but no dated samples from European bison or moose
431 are available during this period to draw comparisons with. Bones of aurochs originating from
432 this highly forested Preboreal and Boreal period decreased mean $\delta^{13}\text{C}$ values to be the lowest
433 among analyzed species in the Early and pre-Neolithic Holocene.

434 In the Early and pre-Neolithic Holocene, the diet of aurochs consisted of a relatively
435 high fraction of grasses and forbs, as reflected by this species having the highest $\delta^{15}\text{N}$ values
436 compared to both moose and European bison diet, which particularly in moose had relatively
437 the highest levels of browse. European bison and moose living in the Early Holocene in more
438 open tundra-like environments (steppe-tundra and forest-steppe with dwarf shrubs and trees
439 such as willows, birches and pines) consumed more easily digestible leaves of shrubs and trees
440 as indicated by nitrogen signatures (Bocherens *et al.*, 2015).

441 During the Neolithic and Late Holocene, the lower $\delta^{13}\text{C}$ values of European bison
442 indicate that this species foraged most frequently in forested habitats, while moose and
443 aurochs utilized this type of habitat less often. Moose exhibited the lowest $\delta^{15}\text{N}$ values of the
444 three species in the Neolithic and Late Holocene, which suggests a diet with a relatively high
445 fraction of browse, whilst European bison and aurochs consumed more herbaceous material.
446 The lowest between the three species, but higher than expected $\delta^{15}\text{N}$ values in moose could be
447 the effect of summer diet enriched with aquatic and herbaceous forage, which is observed in

448 modern moose (Wam & Hjeljord, 2010a). In contrast to our findings, the dental microwear
449 textural analysis (DMTA) showed that during the Late Holocene the diet of aurochs from
450 northeastern Europe was mixed or browsing (Hofman-Kamińska *et al.*, 2018b). This is likely
451 to be the effect of seasonality in the animal's diet, which is possible to detect through teeth
452 microwear analysis (Percher *et al.*, 2018), therefore has a different chronological resolution
453 than the reconstruction of the diet reflecting the whole life of the animal, which is given by the
454 analysis of stable isotopes (Hedges *et al.*, 2007).

455 Stable isotopic compositions show a significant shift of foraging habitats from more
456 open in the Early and pre-Neolithic Holocene to more forested in the Neolithic and Late
457 Holocene specimens in all analyzed species. Such a pattern, already documented in red deer
458 (*Cervus elaphus*) (Drucker *et al.*, 2003, Drucker *et al.*, 2008), is consistent with the changes in
459 the forest cover as a result of vegetation succession in Europe in the Early Holocene, but it is
460 not compatible with the reduction of the forest cover as a result of agricultural activities
461 following the Neolithic and subsequent periods (Woodbridge *et al.*, 2018). Despite reductions
462 in tree cover as a result of Neolithic development, the largest European ungulates continued to
463 forage in forest. This has continued through the last 2000 yrs BP, when the scale of forest
464 reduction was the largest (Kaplan *et al.*, 2009). This probably reflects avoidance of human
465 pressure and the refugial character of forest habitats for large ungulates (Kerley *et al.*, 2012).
466 Thus, aurochs and European bison, pre-adapted to open or mixed habitats (Bocherens *et al.*,
467 2015, Hall, 2008), became classical refugee species *sensu* Kerley *et al.* (2012) after the
468 Neolithic and later transformation of European vegetation. This mechanism is similar to
469 modern examples, where human impact results in a direct loss of habitats and an increase in
470 avoidance behaviour of affected wildlife (Buuveibaatar *et al.*, 2016, Jiang *et al.*, 2007, Paton *et*
471 *al.*, 2017). Modern moose in Scandinavia, as a hunted species, avoid human encounters by
472 exploiting open habitats mostly during the night (Bjørneraas *et al.*, 2011). Conversely, a lack

473 of persecution of modern European bison has resulted in increasing utilization of open habitats
474 by populations that were introduced mainly to forests (Kowalczyk *et al.*, 2013).

475 Stable nitrogen isotope compositions of moose and European bison were lower in the
476 Early and pre-Neolithic Holocene than in the Neolithic and Late Holocene. This may relate to
477 shifts in the diet of these species from more browsing in the Early and pre-Neolithic Holocene
478 to more grazing in the Neolithic and Late Holocene. However, the much lower $\delta^{15}\text{N}$ values in
479 herbivore bones at the beginning of the Holocene in comparison to later periods, might also be
480 connected with lower total available N (g/m^2) in the pioneer stages of soil formation in the
481 glacier forefield, which increases exponentially along the soil developmental gradient
482 (Göransson *et al.*, 2016). Reconstruction of diet based on dental microwear textural analysis
483 (DMTA) showed that European moose had a browsing diet from the Neolithic period through
484 to the Middle Ages. In the case of European bison, the same analysis showed the dominance of
485 graminoids and forbs in the diet of this species living in the Neolithic subalpine region in
486 Switzerland; however, by the Roman period and Middle Ages European bison from
487 northeastern Europe had a mixed diet (Hofman-Kamińska *et al.*, 2018b), probably due to
488 lower accessibility of open habitats. We show that aurochs was the only large herbivore that
489 did not change its $\delta^{15}\text{N}$ values from Early and pre-Neolithic to Neolithic and Late Holocene. It
490 seems that despite the change in environmental conditions and growth of total available N
491 (g/m^2) in soils (Göransson *et al.*, 2016), $\delta^{15}\text{N}$ values in aurochs did not increase. Aurochs diet
492 in the Neolithic and Late Holocene still had a relatively high fraction of graminoids and forbs
493 (similarly to European bison), but due to lower availability of this plant groups in forest
494 habitats, they had to incorporate some woody material into their diet. Thus, the limits set by
495 ecological conditions of forest habitats (i.e. availability of preferred graze) were more
496 profound for specialized grazers such as aurochs than for mixed feeders like European bison.
497 Habitat specialists are more sensitive to environmental changes and vulnerable to extinction

498 (Keinath *et al.*, 2017). This suggests that environmental changes would have had a much
499 stronger impact on aurochs than other species. The extirpation and final extinction in the 17th
500 century of this widely distributed in the Late Pleistocene and Early Holocene species supports
501 this assertion (Wright, 2013, Wright & Viner-Daniels, 2015).

502

503 **Factors influencing $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in large herbivores**

504 Spatio-temporal analysis of stable isotopic ratios of large herbivores throughout the Holocene
505 showed that European bison and aurochs responded to environmental changes, but to different
506 extents with different factors explaining the observed variations. None of the factors
507 considered here influenced $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in Holocene moose bones. Modern studies
508 on moose showed that this species can inhabit a wide variety of habitat types including
509 forests, open willow-birch shrublands bogs, and alder swamps (Gębczyńska & Raczyński,
510 1989, Olsson *et al.*, 2011) and might have a very diverse diet which can contain different
511 types of woody material (e.g. shoots, bark, foliage and fallen leaves) as well as aquatic
512 vegetation, grasses and forbs (Baskin & Danell, 2003, Shipley, 2010, Wam & Hjeljord,
513 2010b), which are characterized by variable nitrogen isotope content (Ben-David *et al.*, 2001,
514 Drucker *et al.*, 2010). An additional factor not tested here that may have had a significant
515 impact on habitat and food type selection (thus influencing the isotopic signatures) is seasonal
516 partial migration of moose (Ball *et al.*, 2001). Lower responses of moose to Holocene
517 environmental changes may also result from a preference for mosaic habitats as mentioned
518 earlier, thus the environmental changes were to some extent beneficial for moose. A second
519 factor is the adaptation and preference of moose to marshlands (Olsson *et al.*, 2011,
520 Stephenson *et al.*, 2006), a habitat which was probably less impacted either by forest
521 succession and human pressure, due to lower accessibility and suitability of these terrains for
522 agriculture activities until drainage in the modern period.

523 Stable carbon and nitrogen isotope variability in European bison and aurochs are best
524 explained by a model incorporating the time factor. Decreasing $\delta^{13}\text{C}$ values through time
525 suggests the shift in the pattern of habitat use from open to more forested habitats. In European
526 bison and aurochs the nonlinear increase of $\delta^{15}\text{N}$ through time from the beginning of the
527 Holocene, reaching the maximum for European bison around 6 ka cal yrs BP and aurochs
528 around 9 ka cal yrs BP, could be an effect of total growth available N (g/m^2) in soils that
529 developed following glacial retreat (Göransson *et al.*, 2016). The second explanation behind
530 this pattern could be changing mean annual temperature, increasing since the beginning of the
531 Holocene up to 7800 cal yr BP (Davis *et al.*, 2003). The $\delta^{15}\text{N}$ in plants increases with
532 increasing mean annual temperature (Amundson *et al.* 2003, Craine *et al.* 2009).

533 The positive relationship between $\delta^{13}\text{C}$ signatures of European bison and aurochs with
534 longitude may indicate more natural pattern of habitat use in a gradient from the south-west to
535 the north-east of the study area related to lower human impact caused by delayed, or less
536 intensive, agricultural development, or lower suitability for cultivation and pasture (Davison
537 *et al.*, 2006, Kaplan *et al.*, 2009). Thus, in the more forested northeastern Europe (Kaplan *et*
538 *al.*, 2009), large ungulates utilized more open habitats, probably having more natural
539 character (open river valleys, large forest gaps caused by disturbance events, such as insect
540 outbreaks or windfalls), which influenced their stable isotope composition. Aurochs were
541 associated with riverine flat-lands (Hall, 2008) and modern European bison restored to forest
542 habitats, but show high preference to open habitats (Kjellander *et al.* in prep) with the lowest
543 $\delta^{13}\text{C}$ values, reflecting utilization of mainly forest habitats at locations with forest cover above
544 70% (Hofman-Kamińska *et al.*, 2018a). The increase of $\delta^{15}\text{N}$ values with longitude suggests a
545 greater contribution of grassy vegetation in the diet of aurochs from northeastern Europe,
546 which confirms our presumptions based on $\delta^{13}\text{C}$ modeling that this species foraged in more
547 natural open habitats in this region.

548 We find that with increasing elevation, $\delta^{15}\text{N}$ values in European bison decrease,
549 reflecting lower $\delta^{15}\text{N}$ values in plants at higher elevations (Huber *et al.*, 2007, Sah &
550 Brumme, 2003, Sparks & Ehleringer, 1997). A similar tendency has been found in the study
551 on modern European bison from Poland, where European bison from the Carpathian
552 Mountains have the lowest $\delta^{15}\text{N}$ among all studied European bison populations (Hofman-
553 Kamińska *et al.*, 2018a). Such an effect has not been detected in aurochs as there is an
554 insufficient range of elevation (Mannel *et al.*, 2007, Sah & Brumme, 2003) available for
555 aurochs samples (0-536 m.a.s.l.), in contrast to European bison for which elevation varied
556 from 32 to 1575 m.a.s.l.

557 Forest cover estimated for analyzed specimen locations shows a hump-shaped
558 polynomial trajectory over time, consistent with changes of forest cover in Europe (Roberts *et*
559 *al.*, 2018). It seems that pattern of habitat use by large herbivore reflected habitat structure
560 shaped by natural and anthropogenic factors (Fyfe *et al.*, 2015). Thus, large herbivores adapted
561 to the environmental changes of the Holocene, expressed some flexibility that promoted their
562 survival in dynamic and often unsuitable conditions of Holocene Europe.

563 Our data show variable shifts in foraging habitats and diet of large herbivores
564 throughout the Holocene in Central and Eastern Europe. These shifts were caused by forest
565 expansion and then increasing human pressure related to the spread of Neolithic agriculture.
566 European bison and aurochs changed from open habitat dwellers to become refugee species in
567 forest habitats. This restriction to less optimal habitats use alongside intensified hunting
568 probably led to lower population densities and reduced fitness of large herbivores, gradual
569 fragmentation of their populations and to their disappearance from large parts of Europe.
570 Moose is the species best adapted to mosaic habitats, and was thus probably less impacted by
571 environmental changes as revealed by models in this study, or may have even benefited from
572 both the expansion of forest and subsequent deforestation, creating more diverse habitats.

573 Some factors (age and longitude) influencing the observed shifts in large herbivore habitat use
574 and diet were similar suggesting a unified pattern of these impacts. Some factors were
575 different (forest cover in aurochs, elevation in European bison) or did not explain the
576 observed variations, which indicate individual responses of each species related to their
577 biology and plasticity. Adaptability of the last remnants of megafauna in Europe allowed
578 them to survive through the entire Holocene (i.e. moose) or until the 17th century as in the
579 case of aurochs (Van Vuure, 2005), or the beginning of the 20th century for European bison
580 (Pucek, 1991). The two survivors that are recolonizing Europe through either natural
581 expansion (moose) or targeted restoration programmes (European bison) are characterized by
582 the highest adaptability to environmental conditions (Hofman-Kamińska *et al.*, 2018a).

583 Patterns analyzed at the European scale may differ regionally and involve factors not
584 considered in this study. We aimed at a synthetic investigation of large herbivore response to
585 environmental changes in the Holocene, which would not be possible at the regional scale due
586 to limited number of specimens. We hope that our data increases broader understanding of
587 animal adaptation to environmental changes and mechanisms of refugee species concept that
588 is widespread in the modern world.

589

590 **ACKNOWLEDGEMENTS**

591 We thank for giving us access to specimens to: L. Costeur (Naturhistorisches Museum Basel,
592 Switzerland), F. E. Zachos (Vienna Natural History Museum, Department of Zoology), U. B.
593 Göhlich (Vienna Natural History Museum, Department of Geology & Palaeontology), M.
594 Nussbaumer (Bern Naturhistorisches Museum, Switzerland), L. Lundqvist (Museum of
595 Zoology, Lund University in Sweden), S. A. Bengtson and K. Berggren (Zoological Museum
596 in Lund, Collection of Zoology and Entomology and Lund University Historical Museum), L.
597 Wickström (Geological Survey of Sweden in Uppsala, Sweden), K. Gregersen (Zoological

598 Museum, Natural History Museum of Denmark in Copenhagen), M. Blant (Swiss Institute for
599 Speleology and Karst Studies), W. Rosendahl (Reiss-Engelhorn-Museen, Abt.
600 Archäologische Denkmalpflege und Sammlungen, Mannheim), N. Spassov (National
601 Museum of Natural History, Sofia), K. Rauscher (Institut für Paläontologie an der Universität
602 Wien, Austria), E. Pucher (Archaeological-Zoological collection at the Natural History
603 Museum Vienna, Austria), U. Schmölcke (Centre for Baltic and Scandinavian Archaeology),
604 D. Krasnodębski (The Institute of Archaeology and Ethnology PAS, Poland), N. Czeremnyh
605 (State Museum of Natural History in Lviv, old Museum Dzieduszyckich, Ukraine), M.
606 Krajcarz (Institute of Geological Sciences PAS in Warsaw), M. Czarniański (Institute of
607 History NAS of Belarus in Minsk), B. Antoniuk (Private Museum in Dobrzyniewo Duże,
608 Poland), W. Litwińczuk (Private Museum of Ethnography and Archeology in Suraz, Poland),
609 M. Szymkiewicz (Nature Museum in Olsztyn, Poland), V. Gedminas (Tadas Ivanauskas
610 Zoological Museum in Kaunas, Lithuania), T. Sawicki and T. Janiak (Museum of the Origins
611 of the Polish State in Gniezno, Poland), A. Juźwiak (Museum in Kwidzyn, Poland), J.
612 Jastrzębski, J. Deptuła (Northern-Mazovian Museum in Łomża, Poland), E. Keczyńska-
613 Moroz (Białowieża National Park, Poland), D. Anatolie, V. Rusu (Institute of Zoology of the
614 Academy of Sciences of Moldova), B. Stachowiak (Museum of the City of Turek named after
615 Józef Mehoffer, Poland), A. T. Halamski, J. Kobylińska (Institute of Paleobiology PAS,
616 Poland), W. Mikucki (Museum of Geology Institute in Warsaw, The Professor Andrzej
617 Myrcha University Centre of Nature, Poland), D. Abłamowicz (District Museum in
618 Sandomierz, Poland), H. Długoszewska – Nadratowska and M. Krajcarz (Mazovian Nobility
619 Museum in Ciechanów, Institute of Geological Sciences, PAS, Poland), D. Serafin (Museum
620 of the Pisz Land), A. Sepioł (Regional Museum in Jasło, Poland), M. Kupczyńska (Faculty of
621 Veterinary Medicine SGGW, Warsaw), A. Archacka (Nature Museum in Drozdowo, Poland),
622 G. Jaworski (Szczecinek Forestry District, Poland), H. Karwowska (Podlaskie Museum in

623 Białystok, Poland), A. M. Hultman and P. Kjellander (Swedish University of Agricultural
624 Sciences, Uppsala, Stjerngranat Museum at Stjärneborg, Sweden), J. Kociuba (Museum in
625 Jarosław, Poland), B. Studencka (Museum of the Earth PAS, Poland), Z. Markovic, S.
626 Alaburic, S. Spasic (Natural History Museum of Belgrad, Serbia), T. Woroncowa-
627 Marcinowska (The Polish Geological Institute - National Research Institute in Warsaw), K.
628 Wysocka (Vinnytsia Regional Local History Museum, Ukraine), Z. Giżejowski (Research
629 Station of Ecological Agriculture and Conservative Animal Breeding PAS in Popielno,
630 Poland), M. Križnar (Slovenian Museum of Natural History), Z. Łonyszyn, L. Chaix
631 (Museum d'Histoire Naturelle, Genève, Switzerland), C. Cupillard (Laboratoire Chrono-
632 Environnement, CNRS-UMR6249, Besançon, France), R. M. Arbogast (CNRS-UMR7044,
633 Strasbourg, France), E. M. Geigl (Institut Jacques Monod, CNRS-UMR7592, Paris, France),
634 Studienzentrum Naturkunde, Universalmuseum Joanneum, Graz (Austria), Lietuvos
635 Nacionalinis Muziejus and Lithuanian Institute of History in Vilnius, and National museum -
636 Palace of the Grand Dukes of Lithuania, and National Museum of Lithuania, Vilnius
637 (Lithuania). We thank Alicja Lasota-Moskalewska and Anna Gręzak for their help in bone
638 identification. We are grateful to Tomasz Kamiński, Paulina Szafrńska and Marcin Churski
639 for their help in sample collection and to Tomasz Borowik for his help in statistical analyzes.
640 The study was financed by the Polish National Science Centre grants no. N N304 301940 and
641 2013/11/B/NZ8/00914 (PI: R. Kowalczyk) and supported by the European Commission's
642 Seventh Framework Programme project No. PIRSES-GA-2009-247652, BIOGEAST and
643 project No. FP7 2010–2013; Agreement No. 245737, BIOCONSUS (Research Potential in
644 Conservation and Sustainable Management of Biodiversity), and Leverhulme Trust (grant
645 number F00568W). Pollen data were extracted from the European Pollen Database
646 (EPD; <http://www.europeanpollendatabase.net/>) and the work of the data contributors and the
647 EPD community is gratefully acknowledged.

648

649 **AUTHORS' CONTRIBUTIONS**

650 EH-K and RK designed the study; EH-K, HB, MP and TS conducted a query in museums;
651 EH-K, HB, RK, WG, DM, MP, GP provided samples; HB and DGD performed stable isotope
652 analyses; RMF and JW provided data and interpretation of forest cover; EH-K performed
653 statistical analyses; EH-K performed graphical visualization; EH-K analyzed the results; EH-
654 K and RK gave interpretations of results; EH-K and RK wrote the original draft, which was
655 reviewed and edited by all co-authors.

656

657 **CONFLICT OF INTEREST**

658 The authors declare no conflict of interest.

659 Supporting information:

660 Table S1. Description of specimen site, inventory number, storage institution, age, stable
661 isotope data and environmental data.

662 Table S2. Model selection (based on the AICc criteria) for the considered linear models of
663 moose, European bison and aurochs.

664 Table S3. Averaged parameter estimates for linear models of moose, European bison, and
665 aurochs data.

666

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