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Comment on "Ecological niche of Neanderthals from Spy Cave revealed by nitrogen isotopes of individual amino acids in collagen." [J. Hum. Evol. 93 (2016) 82-90]

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Collins (2005: 267) We welcome Naito et al's recent efforts to gain greater information about the diet and ecological niche of Neanderthals (Naito, Chikaraishi, *et al.* 2016). However, the application of a relatively novel technique (compound specific amino acid nitrogen isotopic analysis; Amy K. Styring *et al.* 2010; Chikaraishi *et al.* 2014) is not without its problems. The trophic position estimates are given with no associated uncertainty, yet previous studies have found uncertainty of up to ±0.4 (Chikaraishi *et al.* 2011). The reported estimates are based on calculations that use a constant (termed a β value) which is derived from limited studies of terrestrial plant amino acid nitrogen isotopic values (Chikaraishi *et al.* 2011). The β value used in this study may not be a true reflection of plant amino acid nitrogen isotopic variability (Steffan *et al.* 2013; Amy K. Styring *et al.* 2014; Paolini *et al.* 2015; Steffan *et al.* 2015). The authors estimate the trophic position of Neanderthals from Spy Cave as 2.8 (using a β value of -8.4), that is towards the value expected from carnivores reliant solely on protein derived from herbivores. Using other values from published terrestrial C_3 plant amino acid nitrogen isotopic data, we can generate estimates of trophic position ranging from 2.1 (from a β value of -3.3), up to a value of 3.3 (from a β value of -12.1). A trophic position estimate of 2.1 to 3.3 spans a dietary range from individuals who consume predominantly plant protein to those who consume a significant proportion of higher trophic resources – essentially the whole range of speculated Neanderthal diets.(Fuller *et al.* 2010)

However a more critical issue is that of precision and reproducibility in the measurement of the amino acid nitrogen isotopic values themselves. The nitrogen isotopic values of proline and hydroxyproline are not equal in many of the individuals measured in this study (both hominin and animal), in some cases significantly different. Hydroxyproline (Hyp) is essential for the stability of the collagen triple helix: un-hydroxylated recombinant collagen has a significantly lower melting temperature (e.g. Perret *et al.* 2001); and levels of hydroxylation are crudely related to physiological temperature (e.g. Lin & Liu 2006). Hydroxylation occurs via a post-translational modification of Proline (Pro) primarily by collagen prolyl 4-hydroxylase (resulting in 4-hydroxyproline) although some residues hydroxylated at the 3-H position by proline 3-hydroxylase (Gorres & Raines 2010). Both enzymes hydroxylate collagen of the assembled propeptide in the lumen of the endoplasmic reticulum prior to folding into the triple helix. Proline has only one nitrogen, an essential component of the peptide bond. Because hydroxylation occurs after sequence assembly, the nitrogen in Hyp originates from Pro. There is should be no isotopic difference in nitrogen between the amino acid (Pro) and its post-translationally modified variant (Hyp) and the most parsimonious explanation for any observed difference is measurement error.

Collagen is the protein most often used in archaeological isotopic studies, with a high abundance of Hyp (in endotherms approximately 50% of collagen Pro residues are hydroxylated); consequently a cross plot of Pro *vs.* Hyp offers an opportunity for analytical quality assessment. We have compared available collagen nitrogen isotopic data derived from Pro and Hyp. Measurements carried out in the JAMSTEC laboratory by GC-C-IRMS use the method of *N*-pivaloyl/isopropyl derivatization (Metges *et al.* 1996), whilst the Bristol laboratory uses N-acetyl-i-propyl derivatization prior to GC-C-IRMS (Amy K. Styring *et al.* 2012). The values of Hyp and Pro measured at Bristol are equal, as expected from the known biochemistry, but measurements from JAMSTEC, including those from this study, deviate significantly from the 1:1 line (Fig 1).

Whilst Pro and Hyp nitrogen isotopic values were not used in the trophic position estimates generated here, the difference in their measured values casts some doubt on the nitrogen isotopic measurements of all amino acids published in this paper. Furthermore, there have been suggestions that better trophic position estimates could be derived from compound-specific nitrogen isotopic analyses of multiple amino acids, including Pro, so any observed differences between the Pro-Hyp pair could be problematic for such future analyses (Nielsen *et al.* 2015).

We urge caution in the interpretation of data based on measurements that are potentially flawed, be they from extinct hominins or other humans or animals (Naito, Honch, *et al.* 2010; Naito, Chikaraishi, *et al.* 2010; Naito, Chikaraishi, Ohkouchi, & Yoneda 2013; Naito, Chikaraishi, Ohkouchi, Drucker, *et al.* 2013; Itahashi *et al.* 2014; Naito *et al.* 2016/4; Naito, Chikaraishi, *et al.* 2016; Naito, Germonpré, *et al.* 2016).

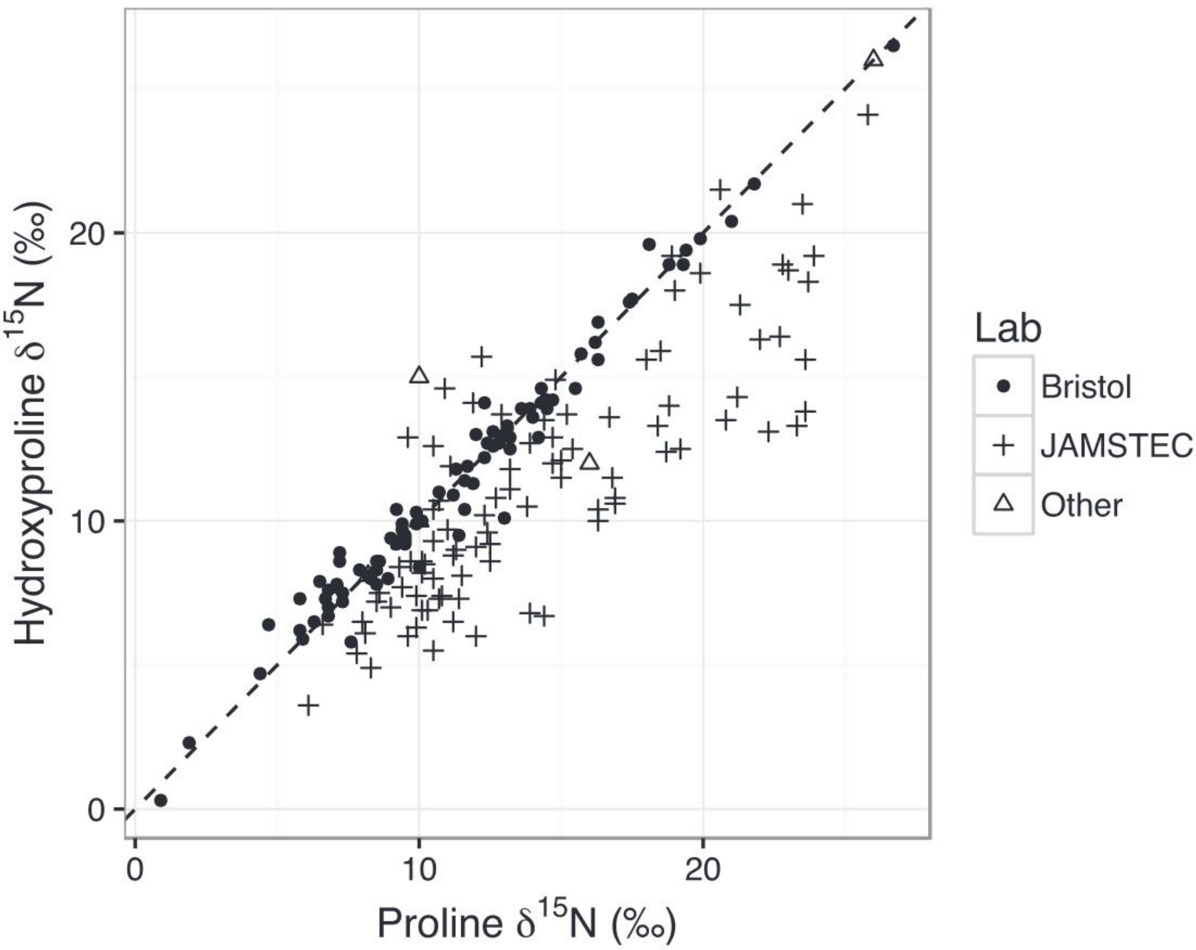

Figure 1: Comparison of collagen proline and hydroxyproline δ^{15}N from known studies.

Dashed line marked is the x=y line, as would be expected based on biochemical pathways. JAMSTEC data: N=110, measured using GC-C-IRMS after *N*-pivaloyl/isopropyl (Pv/iPr) derivatization (Naito, Chikaraishi, *et al.* 2010; Naito, Honch, *et al.* 2010; Naito, Chikaraishi, Ohkouchi, & Yoneda 2013; Naito, Chikaraishi, Ohkouchi, Drucker, *et al.* 2013; Itahashi *et al.* 2014; Naito *et al.* 2016/4; Naito, Chikaraishi, *et al.* 2016; Naito, Germonpré, *et al.* 2016). Bristol data, N=87, measured using GC-C-IRMS after N-acetyl-i-propyl derivatization (O'Connell unpubl data; Amy K. Styring *et al.* 2010; Amy Keita Styring 2012). Other data, N=6, measured using ion exchange chromatography, offline combustion and subsequent IRMS measurement (Hare *et al.* 1991; Hare & Estep 1983; Tuross *et al.* 1988). Regression equation for JAMSTEC: y= 0.90x +4.07; R^2 =0.79. Regression equation for Bristol: y= 1.01x – 0.10; R^2 =0.97.

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