# Manuscript Details

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**McDonald Institute for Archaeological Research**

Founder: Dr D.M. McDonald
Director : Professor Graeme Barker FBA

Downing Street, Cambridge, CB2 3ER, UK

From Dr Tamsin O'Connell
Tel: 01223-339344
Fax: 01223-333536
tco21@cam.ac.uk

**Ref: HUMEV_2016_27R1 – Response to copy editor's comments**

10th May 2017

Dear Professor Plavcan,

Please find enclosed the copy edited version of our comment on Naito et al. JHE 2016.

It is now clean, as I accepted all the changes that I wanted to accept. But I had some questions for three of the suggested edits, and wanted to make sure that you knew that I did not accept all of them.

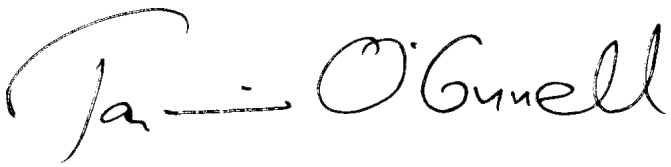
On P4, for the sentence "Thus, Naito et al.'s (2016a) conclusion that Neanderthals "could rely on plants for up to ~20% of their protein source" seems premature", I didn't put a page number in, but made it clear that their claim was from the abstract. It now reads: "Thus, Naito et al.'s (2016a) statement in their abstract that Neanderthals "could rely on plants for up to ~20% of their protein source" seems premature."

On P9 (Ref list), the copy editor changed J. Quat. Sci to J. Quaternary Sci. (for Naito et al 2016b reference), but the LWTA abbreviation of Quaternary is Quat, so I thought I was supposed to use the abbreviation. I have left it as J. Quat. Sci.

On P10 (Ref list), the copy editor asked if "Rapid Commun." was needed, and the answer is yes, because that is part of the name of the journal "Rapid Communications in Mass Spectrometry".

I hope all this is clear.

Yours sincerely,



Dr Tamsin O'Connell

Senior Lecturer in Archaeological Science
Department of Archaeology & Anthropology

# 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]

Tamsin C. O'Connell [a *] and Matthew J. Collins [b,c]

[a] Department of Archaeology & Anthropology, University of Cambridge, Downing Street, Cambridge, CB2 3DZ, United Kingdom.

email: tco21@cam.ac.uk

[b] EvoGenomics, Natural History Museum of Denmark, University of Copenhagen, Sølvgade 83, Copenhagen 1307 S, Denmark.

[c] BioArCh, Environment Building, Department of Archaeology, University of York, Wentworth Way, Heslington York YO10 5NG, United Kingdom.

email: matthew@palaeome.org

* corresponding author = TCO'C

email: tco21@cam.ac.uk

Telephone: +44-1223-339344

Fax: +44-1223-333536

**Keywords:** compound-specific nitrogen isotope analysis, ecological niche, trophic position, trophic discrimination factor, proline, hydroxyproline

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have influenced its outcome.

We welcome Naito et al.'s recent efforts to gain greater information about the diet and ecological niche of Neanderthals, through compound-specific amino acid nitrogen isotopic analysis of bone collagen from Neanderthal remains from Spy Cave in Belgium as well as of specimens of contemporary animal species (Naito et al., 2016a). The application of a relatively novel technique (see: Styring et al., 2010; Chikaraishi et al., 2014; McMahon and McCarthy, 2016) is not without its problems, and we would like to provide a critical comment on the implications of uncertainties for the interpretation and application of this method, and on analytical aspects of this technique.

The trophic position estimates are given with no assessment of error, yet previous studies have found associated uncertainty of up to ±0.4 (Chikaraishi et al., 2011). The reported estimates are based on calculations that use two constants, one termed a β value, and one termed a trophic discrimination factor or TDF (also known as the trophic enrichment factor TEF): in Equation 1 in Naito et al. (2016a), the β value is labelled as such, and the TDF is given as 7.6‰. In a systematic review of compound-specific amino acid nitrogen isotopic analysis in marine consumers, Nielsen et al. (2015) found that the uncertainty in trophic position estimates was most influenced by changes in the TDF. They suggested that a TDF value of 7.6‰ is too high for marine animals, and may be species-specific (as was also found by McMahon and McCarthy, 2016). Furthermore, Nielsen et al. (2015) found that the estimated β value from their meta-analysis was lower than that proposed by Chikaraishi et al. (2009) for marine foodwebs. Whilst this is not directly relevant to the current study of (mostly) terrestrial-source feeding animals (i.e. Neanderthals), it does suggest that more work is needed to understand the accuracy and precision of this method to estimate trophic position.

Naito et al. (2016a) do discuss the possible impact of TDF variability on the trophic position estimates, but they do not question the validity of the β value used. The

estimates reported in their paper use a β value derived from limited studies of terrestrial plant amino acid nitrogen isotopic values (Chikaraishi et al., 2011), which may not be a true reflection of plant amino acid nitrogen isotopic variability (Steffan et al., 2013, 2015; Styring et al., 2014; Paolini et al., 2015). The authors estimate the trophic position of Neanderthals from Spy Cave as 2.8 (using a β value of -8.4‰), which 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‰: Paolini et al., 2015), up to a value of 3.3 (from a β value of -12.1‰: Styring et al., 2014). 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 very high proportion of higher trophic resources – essentially the whole range of speculated Neanderthal diets. Thus, Naito et al.'s (2016a) statement in their abstract that Neanderthals "could rely on plants for up to ~20% of their protein source" seems premature.

A more critical issue, however, is that of precision and reproducibility in the measurement of the amino acid nitrogen isotopic values themselves. The nitrogen isotopic values of proline (Pro) and hydroxyproline (Hyp) are not equal in many of the individuals measured in this study (both hominin and other animal), and in some cases are significantly different. Hydroxyproline 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 and Liu, 2006). Hydroxylation occurs via a post-translational modification of proline primarily by collagen prolyl 4-hydroxylase (resulting in 4-hydroxyproline) although some residues are hydroxylated at the 3-H

position by proline 3-hydroxylase (Gorres and 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, which is an essential component of the peptide bond. Because hydroxylation occurs after sequence assembly, the nitrogen in Hyp originates from Pro. There 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. The JAMSTEC laboratory uses the method of N-pivaloyl-i-propyl derivatization prior to GC-C-IRMS (Metges et al., 1996), whilst the Bristol laboratory uses N-acetyl-i-propyl derivatization prior to GC-C-IRMS (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: no details are given as to the composition of the reference amino acid mixture analysed, and there are no uncertainties reported for any of the individual amino acid measurements (Table S4 in the Supplementary Online Material of Naito et al. 2016a). 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 et al., 2010a, 2010b, 2013a, 2013b, 2016a, 2016b, 2016c; Itahashi et al., 2014).

## Acknowledgements

We would like to thank the editor and three anonymous referees for useful comments on this piece.

## Figure Caption

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

Dashed line 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-i-propyl derivatization (Naito et al., 2010a, 2010b, 2013a, 2013b, 2016a, 2016b, 2016c; Itahashi et al., 2014). Bristol data: *n*=87, measured using GC-C-IRMS after N-acetyl-i-propyl derivatization (O'Connell unpublished data; Styring et al., 2010; Styring, 2012). Other data: *n*=6, measured using ion exchange chromatography, offline combustion and subsequent IRMS measurement (Hare and Estep, 1983; Tuross et al., 1988; Hare et al., 1991). 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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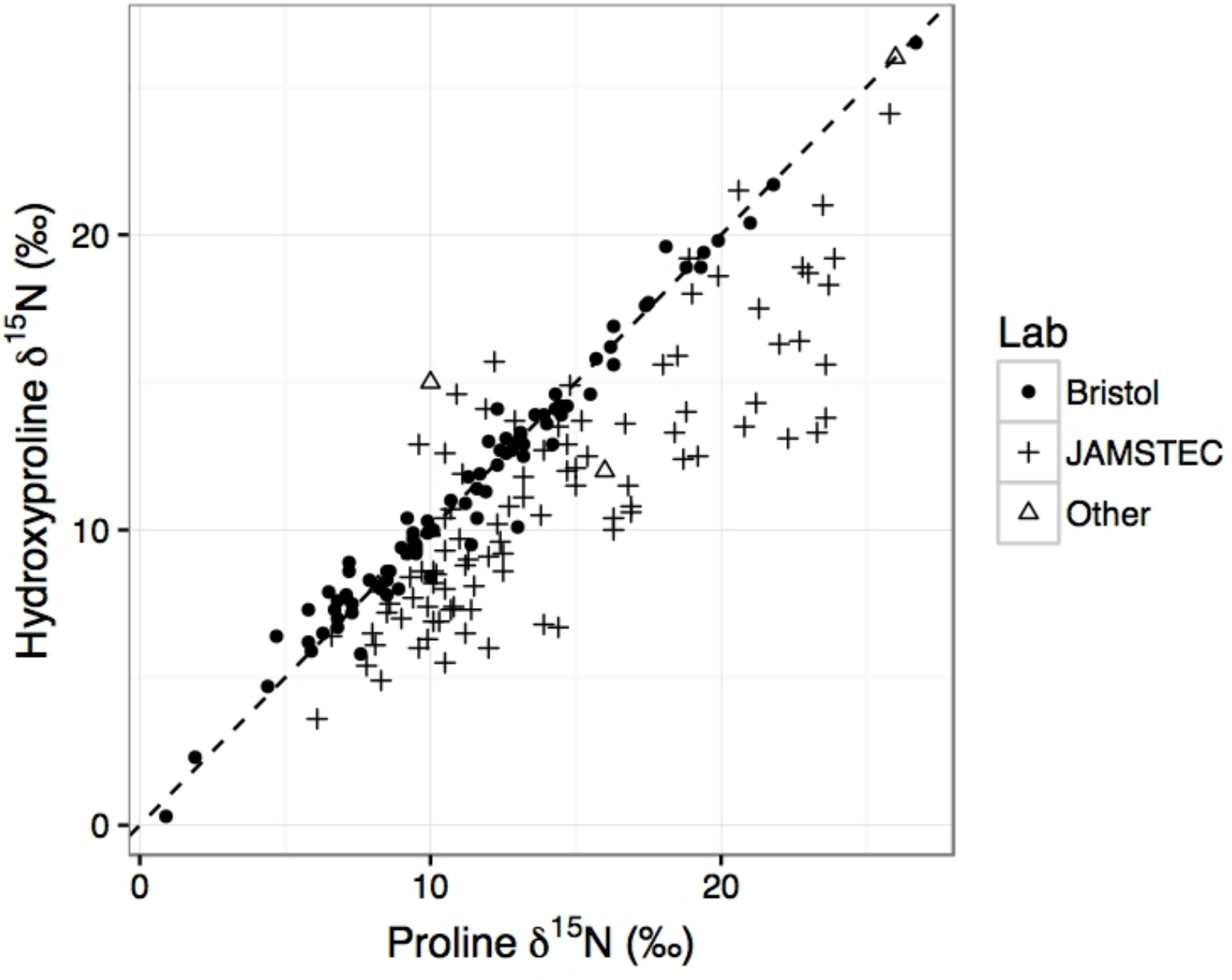
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