



## Research

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# Stable isotope analyses of amino acids reveal the importance of aquatic resources to Mediterranean coastal hunter–gatherers

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Determining the degree to which humans relied on coastal resources in the past is key for understanding long-term social and economic development, as well as for assessing human health and anthropogenic impacts on the environment. Prehistoric hunter–gatherers are often assumed to have heavily exploited aquatic resources, especially those living in regions of high marine productivity. For the Mediterranean, this view has been challenged, partly by the application of stable isotope analysis of skeletal remains which has shown more varied coastal hunter–gatherer diets than in other regions, perhaps due to its lower productivity. By undertaking a more specific analysis of amino acids from bone collagen of 11 individuals from one of the oldest and best-known Mesolithic cemeteries in the Mediterranean, at El Collado, Valencia, we show that high levels of aquatic protein consumption were achieved. By measuring both carbon and nitrogen in amino acids, we conclude that some of the El Collado humans relied heavily on local lagoonal fish and possibly shellfish, rather than open marine species. By contrast to previous suggestions, this study demonstrates that the north-western coast of the Mediterranean basin could support maritime-oriented economies during the Early Holocene.

## 1. Introduction

Once considered marginal to prehistoric human societies, coastal environments have become increasingly central in archaeological theories [1,2], with studies demonstrating their role in early human dispersal around the continents [3,4], in fuelling population growth, complex forms of social organization [5–7], the emergence and spread of early food production systems [8–10]. Nevertheless, the degree to which coastal Mediterranean hunter–gatherers relied on aquatic resources remains contentious. It is often thought that exploitation of marine taxa broadened and intensified in the Holocene through the development of specialized fishing and shellfish gathering and new technologies [11,12] thereby encouraging sedentism and surplus production. The counterargument is that coastal environments were heavily exploited well before the Holocene, but that much of the evidence has been lost due to rising sea levels [3]. The extent to which regional variation in marine productivity and thus the relative availability of coastal resources influenced the development of hunter–gatherer subsistence economies is also unclear. It is often assumed that the Mediterranean basin, due to its relatively low primary productivity, low tidal amplitude and diminished intertidal zone [13], led to

a different dietary adaptation compared to the maritime-focused hunter-gatherer-fisher economies that developed along the Atlantic and Baltic coasts. Certainly, the frequency of coastal shell middens is lower. Yet, the remains of marine mammals, fish and molluscs are frequently found on Mediterranean Mesolithic coastal sites, sometimes dominating the assemblages (e.g. [14,15]), suggesting, at the very least, they were a key strategic supplement to terrestrial-based subsistence [16–19].

Taphonomy biases adversely affecting the preservation and recovery of marine fish bone and plant remains prevents a more precise assessment of the relative contribution of marine and terrestrial foods. For this reason, stable carbon ( $\delta^{13}\text{C}_{\text{coll}}$ ) and nitrogen isotopes ( $\delta^{15}\text{N}_{\text{coll}}$ ) of human bone collagen have provided a useful tool for directly investigating spatial and temporal dietary variation among coastal hunter-gatherers. Indeed, such analysis supports a much greater reliance on marine resources by Late Mesolithic Atlantic and Baltic hunter-gatherers compared to those from the Mediterranean. For example, in Atlantic contexts from Britain, France and Portugal, analysis of Late Mesolithic skeletal remains show a sustained and heavy reliance on marine foods, particularly fish, estimated to contribute at least half their total dietary protein and up to 90% [20]. By contrast, analyses of Upper Palaeolithic and Mesolithic skeletal remains from the Mediterranean reveal diets where the majority of protein is derived from the terrestrial hunted game [15,17,18,21–30], even at sites where fish remains dominate the faunal assemblage [15,19,28,29,31]. From these data it is argued that the availability of marine foods from the Mediterranean may have been, in general, too low to sustain human populations, leading to the development of a broader, mixed marine-terrestrial strategy and perhaps greater terrestrial mobility [29].

Nevertheless, the accuracy and confidence for quantifying the marine contribution to diet using stable isotopes of bone collagen have been questioned. Concerns have centred around the ability to detect low but significant marine consumption (e.g. <20% by dry weight [32]). As collagen is synthesized from different dietary macronutrients [33] (i.e. protein, carbohydrate and lipids), it is proposed that a mixed diet derived from different types of marine and terrestrial foods is not always easily decipherable from bulk collagen stable isotope values. Also, without knowledge of the isotope values of baseline primary producers, which may vary spatially, it is difficult to make accurate comparisons between regions. This is particularly relevant to the Mediterranean region where the range of collagen  $\delta^{15}\text{N}$  values ( $\delta^{15}\text{N}_{\text{coll}}$ ) of marine fish from archaeological sites is typically lower than those from Atlantic sites [22,34] and often overlaps with those of terrestrial mammals [35]. Carbon and nitrogen compound-specific isotope analysis (CSIA) of individual collagen amino acids (CSIA-AA) is being increasingly applied to circumvent these issues, as amino acids can be traced to dietary sources with more certainty and independently assess baseline  $\delta^{15}\text{N}$  values and trophic position (e.g. [36–39]). This approach has been used to investigate intra-site dietary differences in marine consumption with more accuracy and precision than bulk isotope analysis [40].

Here, we apply CSIA-AA to one of the largest and most important Mesolithic human bone assemblages from the Mediterranean coast; the site of El Collado (Oliva, Valencia,

Spain) [41,42]. Previous bulk collagen isotope analyses of nine individuals from this site, showed variability in the extent of marine consumption; some individuals had entirely terrestrial diets whereas it was suggested that others obtained approximately 25% of total dietary protein from marine sources [29]. This assessment was based on the linear interpolation between endpoints estimated from terrestrial and marine fauna. Subsequent to this important finding, it was shown that although  $\delta^{13}\text{C}_{\text{coll}}$  values clearly discriminate between marine and terrestrial Mediterranean fauna,  $\delta^{15}\text{N}_{\text{coll}}$  are more difficult to interpret since the marine and terrestrial endpoints for the Mediterranean are more similar [34]. The objective of this study was therefore to extend the sampling of human remains from El Collado and to establish whether the CSIA can more accurately quantify the marine contribution by considering the  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of individual amino acids obtained from bone collagen. As one of only very few Late Mesolithic burial grounds in the Western Mediterranean, furthermore detailed dietary analysis has the potential to provide an almost unique insight into food consumption practices during a key period in Mediterranean prehistory and one that dates immediately prior to the arrival of farmers, with further implications for agricultural adoption.

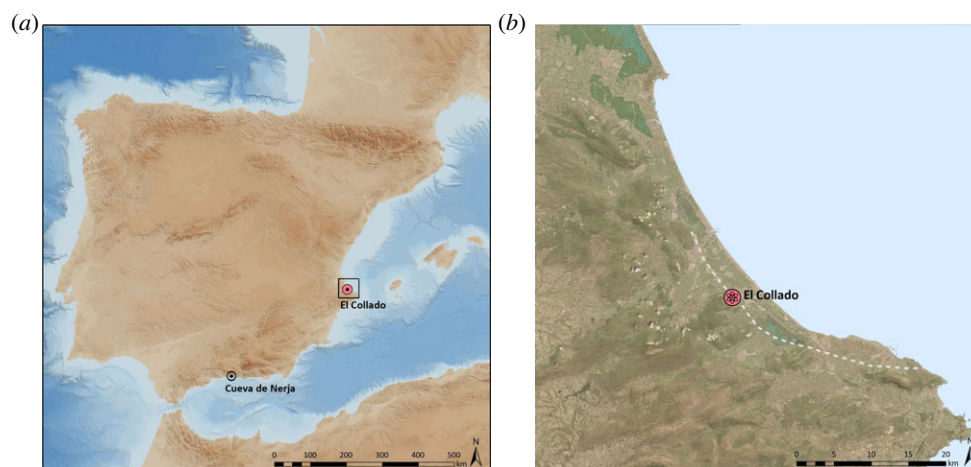
### (a) The El Collado site

El Collado is located in Oliva about 7 km from the modern Mediterranean coastline in the Gulf of Valencia, Spain (figure 1). It is an open-air site on a hillside, sheltered by a limestone cliff, about 10 m above sea level. During the Mesolithic, the lowland around the site consisted of coastal freshwater swamps and marshes, and brackish lagoon biotopes [43], following a rise in sea level from about 8300 BP reaching a maximum at 6130 BP. Excavations in the late 1980s revealed a total of 14 graves (COLL 1–14), 13 of which were primary burials attributed to the Mesolithic period. A secondary burial (COLL 9) contained only a skull and an isolated skull was also recovered close to burial 12 (COLL 12). AMS dates of 10 of the individuals showed that the site was occupied, possibly in phases, from *ca* 9500 cal BP to 8500 cal BP making it one of the oldest known cemeteries on the Iberian Peninsula [42]. Furthermore, the cemetery is clearly connected to a habitation site consisting of a substantial mixed marine-terrestrial shell midden, with lithic tools and other faunal remains, including a fish assemblage dominated by the gilthead sea bream (*Sparus aurata*) [17].

## 2. Material and methods

### (a) Sampling

In this study, 11 individuals from the 1987–1988 excavations were analysed with permission provided by the Museu de Prehistòria de València (Valencia, Spain). The original osteological report [44] was reassessed during the sampling procedure, and the sex and age-at-death of the 11 individuals were refined including updated osteoarchaeological methods [45–47] (electronic supplementary material, table S1). The sampling process was performed carefully by selecting, whenever possible, fragments of long bone diaphysis from the lower limbs. In conjunction with the analysis of human remains, seven faunal remains from El Collado (four terrestrial herbivores and three marine fish) were analysed (electronic supplementary material, table S1). To



**Figure 1.** (a) Map of the Iberian Peninsula showing the location of the sites of El Collado (red) and Cueva de Nerja; and (b) the location of the El Collado site in the Southern Sector of the Valencian Gulf, Spain, with the representation of the position of the Flandrian coastline  $6130 \pm 100$  BP (dashed line), showing a sea level 2 m higher and about 3 km inwards from the current position of the coastline, based on [17].

increase the faunal reference further, collagen extraction was attempted from 13 marine fish bones from Mesolithic ( $n = 7$ ) and Early Neolithic ( $n = 6$ ) layers at the sites of Cueva de Nerja (Malaga, Spain), chosen for its proximity in time and space to El Collado.

### (b) Collagen extraction

Collagen was extracted and prepared for the stable isotope analysis at the BioArCh laboratories in the Department of Archaeology, University of York (UK), using previously published approaches [48]. Briefly, the bone samples were cleaned using a sandblaster (80 060 Master Problast 3) to remove potential post-depositional contamination. Shards of bones (approx. 200 to 300 mg) were demineralized using 0.6 M HCl at 4°C for several days, then rinsed with ultrapure H<sub>2</sub>O (milli-Q) and gelatinized with 0.001 M HCl at 80°C for 48 h. The supernatant containing the collagen was filtered using polyethylene Ezee filters (Elkay Laboratories, 9 ml, pore size 60–90 µm) and then 30 kDa Amicon Ultra-4 centrifugal filter units (Millipore, MA, USA). Samples were then frozen for 24–48 h at –20°C, lyophilized, and weighed into tin capsules (0.5 mg per duplicate) for stable isotope analysis.

### (c) Bulk EA-IRMS

The measurement of the stable carbon and nitrogen isotope ratios was performed in duplicate in the BioArCh laboratories, Department of Archaeology at the University of York, using a Sercon EA-GSL elemental analyser coupled to a Sercon 20–22 continuous flow isotope ratio mass spectrometer (Sercon, Crewe, UK). This measures the ratios of  $^{13}\text{C}:^{12}\text{C}$  and  $^{15}\text{N}:^{14}\text{N}$  relative to a standard (V-PDB for carbon and AIR for nitrogen) and expresses the stable isotope values in delta notation ( $\delta$ ) in parts per mil (‰). The accuracy of the measurements was determined according to Kragten [49] by combining uncertainties in the values of standard reference materials and sample replicates within each analytical run. These were international standards IAEA 600 (caffeine:  $\delta^{13}\text{C}_{\text{raw}} = -27.59 \pm 0.08\text{‰}$ ,  $\delta^{13}\text{C}_{\text{true}} = -27.77 \pm 0.043\text{‰}$ ,  $\delta^{15}\text{N}_{\text{raw}} = 1.02 \pm 0.08\text{‰}$ ,  $\delta^{15}\text{N}_{\text{true}} = 1 \pm 0.2\text{‰}$ ), IA CANE (sugar cane:  $\delta^{13}\text{C}_{\text{raw}} = -11.64 \pm 0.13\text{‰}$ ,  $\delta^{13}\text{C}_{\text{true}} = -11.64 \pm 0.03\text{‰}$ ), IAEA N2 (ammonium sulfate:  $\delta^{15}\text{N}_{\text{raw}} = 20.17 \pm 0.06\text{‰}$ ,  $\delta^{15}\text{N}_{\text{true}} = 20.3 \pm 0.2\text{‰}$ ) and an internal standard (fish gelatine:  $\delta^{13}\text{C}_{\text{raw}} = -15.43 \pm 0.14\text{‰}$ ,  $\delta^{13}\text{C}_{\text{true}} = -15.32 \pm 0.03\text{‰}$ ,  $\delta^{15}\text{N}_{\text{raw}} = 15.01 \pm 0.09\text{‰}$ ,  $\delta^{15}\text{N}_{\text{true}} = 15.2 \pm 0.12\text{‰}$ ).

In addition, a bovine bone control was extracted and analysed within the same batch, producing the following average

values ( $\delta^{13}\text{C} = -22.97 \pm 0.10\text{‰}$ ,  $\delta^{15}\text{N} = 5.85 \pm 0.17\text{‰}$ ). This was within the overall mean value from 50 separate extracts of this bone sample, which produced values of  $\delta^{13}\text{C} = -22.93 \pm 0.20\text{‰}$  and  $\delta^{15}\text{N} = 6.15 \pm 0.29\text{‰}$ .

### (d) Compound-specific stable isotope measurements of amino acids

The isotopic composition of amino acids was analysed in 11 human collagen samples and five faunal collagen samples (two bovinds, two cervids and one fish) from El Collado, and five fish collagen samples from Cueva de Nerja. Collagen extracts were hydrolysed (6 M HCl, 200 µl, 110°C, 24 h) after addition of 50 µl of an internal norleucine standard (Sigma-Aldrich) of known isotopic composition. The hydrolysates were centrifuged (11 000g, 1 min) using Pall Nanosep filters (0.45 µm) to remove the remaining insoluble material. The hydrolysates were gently dried at room temperature under N<sub>2</sub>, redissolved in 0.1 M HCl (100 µl), and stored at –20°C until required for analysis.

Amino acids were then derivatized to form *N*-acetyl-*i*-propyl (NAIP) esters [50]. Briefly, isopropanol and acetyl chloride (1 ml; 4:1 v/v) were added to the dried samples, and the tubes were sealed and heated at 100°C (1 h). After 1 hour, sample mixtures were cooled (at –20°C), and the solution was dried under a gentle stream of N<sub>2</sub>. Dichloromethane (DCM) was added (2 × 0.5 ml) and blown down under a gentle stream of N<sub>2</sub> to remove excess reagents. Next, a mixture of acetic anhydride, triethylamine and acetone (1 ml; 1:2:5, v/v/v) was added to the tubes and heated at 60°C (10 min). The mixture was cooled and evaporated to dryness under a gentle stream of N<sub>2</sub>. NAIP esters were then dissolved in ethyl acetate (EtAc; 2 ml). A saturated NaCl solution (1 ml) was added to separate polar and/or inorganic components, and the organic phase was transferred into a new culture tube. The phase separation was repeated with additional EtAc (1 ml). Trace amounts of water were removed with molecular sieves (sodium aluminium silicate, 0.3 nm: Merck KGaA, Darmstadt, Germany). The EtAc containing the NAIP esters was blown down under a gentle stream of N<sub>2</sub>, and then DCM (1 ml) was added and dried to remove excess water. Samples were redissolved in known quantities of EtAc and stored at –20°C until required for analysis by GC-C-IRMS. The same derivatization procedure was used for preparing mixtures of international reference standards (Indiana, USA and SHOKO Science, Japan) and standards purchased from Sigma-Aldrich (Sigma-Aldrich Company, UK).

The GC-C-IRMS measurements of the amino acids were conducted using a Delta V Plus IRMS (Thermo Fisher Scientific, Bremen, Germany) linked to a Trace Ultra gas chromatograph (Thermo Fisher Scientific, Bremen, Germany) with a GC IsoLink II interface fitted with a Cu/Ni combustion reactor maintained at 1000°C. Ultrahigh-purity-grade helium with a flow rate of 1.4 ml min<sup>-1</sup> was used as the carrier gas, and parallel acquisition of flame ionization data were achieved by diverting a small part of the flow to an integrated flame ionization detector (Thermo Fisher Scientific). Ethyl acetate was used to dilute the samples, and 1 µl of each sample and 2 µl of each standard were injected at 240°C with a 3.5 s pre-injection dwell time onto a custom DB-35 fused silica column (60 m × 0.32 mm × 0.50 µm; Agilent J&W Scientific Technologies, Folsom, CA, USA). All samples were injected in triplicate. The oven temperature program used for samples and standards was as follows: 40°C (hold 5 min) and then increasing by 15°C min<sup>-1</sup> up to 120°C, then by 3°C min<sup>-1</sup> up to 180°C, then by 1.5°C min<sup>-1</sup> up to 210°C, then by 5°C min<sup>-1</sup> up to 280°C (hold 8 min). A Nafion membrane removed water, and a cryogenic trap was used to remove CO<sub>2</sub> from the oxidized and reduced sample when operated in nitrogen mode. In carbon mode, eluted products were combusted to CO<sub>2</sub> and ionized in a mass spectrometer by electron impact. Ion intensities of mass/charge ratio (*m/z*) 44, 45 and 46 were monitored to automatically compute the <sup>13</sup>C:<sup>12</sup>C ratio of each peak in the samples. In nitrogen mode, ion intensities of *m/z* 28, 29 and 30 were monitored to automatically compute the <sup>15</sup>N:<sup>14</sup>N ratio of each peak in the samples. Computations were made with Isodat (v. 3.0; Thermo Fisher Scientific) and were based on comparisons with a repeatedly measured high-purity standard reference gas (CO<sub>2</sub> or N<sub>2</sub>). The results from the analysis are reported in parts per mil (‰) relative to international standards using the  $\delta$  notation.

### (e) $\delta^{15}\text{N}$ measurements of amino acids

The  $\delta^{15}\text{N}$  values reported in the present are the mean of triplicate  $\delta^{15}\text{N}$  measurements. An amino acid international standard mixture of known isotopic composition was run after every three sample injections to monitor instrument performance and drift. The amino acid standard mixture used for  $\delta^{15}\text{N}$  determinations comprised eight international standards (Indiana and SHOKO Science) and L-norleucine (Sigma-Aldrich).  $\delta^{15}\text{N}$  true values of L-norleucine were determined in-house by EA-IRMS. International standard average raw values and s.d. across the runs were as follows: Ala, 44.46 ± 1.66‰ (true: +43.25 ± 0.07‰); Gly, 3.44 ± 1.49‰ (true: +1.76 ± 0.06‰); Val, -4.30 ± 0.98‰ (true: -5.21 ± 0.05‰); Leu, 7.83 ± 1.29‰ (true: 6.22‰); Ile, +15.68 ± 0.78‰ (true: +14.55 ± 0.23‰); Asp, 35.03 ± 0.16‰ (true: 35.2‰); Glu, -3.76 ± 0.43‰ (true: -4.52 ± 0.06‰); Hyp, -8.73 ± 0.44‰ (true: -9.17‰); Phe, 1.82 ± 0.32‰ (true: 1.70 ± 0.06‰). Sample  $\delta^{15}\text{N}$  raw values were corrected by the calibration curve and the L-norleucine internal standard true value.

### (f) $\delta^{13}\text{C}$ measurements of amino acids

The  $\delta^{13}\text{C}$  values reported are a mean of triplicate  $\delta^{13}\text{C}$  measurements. Amino acids in the samples were first corrected for the isotopic difference between L-norleucine in the standard mixture and L-norleucine in the sample.  $\delta^{13}\text{C}$  amino acid measurements were then corrected by specific correction factors to account for the derivatizing carbon and the kinetic isotope effect [51]. A standard amino acid mixture was run after every three sample injections, and the average correction factors from the standard mixture were used for the correction of the samples (Sigma-Aldrich, UK). True  $\delta^{13}\text{C}$  values of standards were measured by EA-IRMS. The standard  $\delta^{13}\text{C}$  average correction factor values and SD across the runs were as follows: Ala, -30.15 ± 1.28‰ (true: -19.31 ± 0.02‰); Gly, -35.02 ± 1.65‰ (true: -33.31 ±

0.02‰); Val, -25.35 ± 1.83‰ (true: -10.89 ± 0.02‰); Leu, -24.67 ± 1.64‰ (true: -13.78 ± 0.06‰); Ile, -31.57 ± 1.73‰ (true: 24.89 ± 0.07‰); Nle, -31.81 ± 1.85‰ (true: -27.59 ± 0.02‰); Thr, -31.40 ± 1.67‰ (true: -10.46 ± 0.01‰); Ser, -33.54 ± 1.50‰ (true: -12.54 ± 0.09‰); Pro, -23.72 ± 1.66‰ (true: -12.33 ± 0.02‰); Asp, -30.60 ± 1.71‰ (true: -27.52 ± 0.12‰); Met, -33.98 ± 2.12‰ (true: -29.88 ± 0.14‰); Glu, -30.17 ± 1.67‰ (true: -28.57 ± 0.09‰); Hyp, -29.79 ± 1.56‰ (true: ); Phe, -20.21 ± 1.43‰ (true: -11.52 ± 0.05‰); Lys, -28.80 ± 1.88‰ (true: -13.7 ± 0.11‰); Tyr, -32.19 ± 1.30‰ (true: -24.85 ± 0.02‰). Correction factors induce a new source of error; therefore, the error propagated for each amino acid was calculated according [51].

### (g) CSIA-AA quality control criteria

We calculated the theoretical collagen  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values based on their amino acid values. The amino acids measured by GC-C-IRMS represent 90.4% and 80.69% of total carbon and nitrogen, respectively in mammalian collagen. We calculated the estimated bulk collagen  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values by mass balance equations considering the relative contribution of each amino acid to collagen and compared the obtained values with those measured via EA-IRMS as previously detailed [40]. Generally, we excluded samples where the estimated observed offset was greater than 2 $\sigma$  of the mean value which included all the  $\delta^{13}\text{C}$  values measured on the terrestrial fauna and two of the humans (Coll 5 and Coll 8). We observed a consistent  $\Delta^{13}\text{C}_{\text{est-obs}}$  offset for the archaeological fish remains from Cueva de Nerja (3.9 ± 0.7‰) which might relate to substantial amounts of lipid co-extracted in these extracts that are reducing the bulk collagen  $\delta^{13}\text{C}$  value (80). We have therefore included these data in figure 3 where they plot in the range of other marine fish samples. The mean  $\Delta^{15}\text{N}_{\text{est-obs}}$  of all the samples used in this study was 0.22‰ (±0.81‰). We also monitored the relationship between proline and hydroxyproline stable isotope values which should be highly correlated due to similarities in the way these amino acids are biosynthesized [52]. The Pearson's product-moment correlation between the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of proline and hydroxyproline from this study were  $R=0.988$  for  $\delta^{15}\text{N}$  ( $t=28.563$ , d.f. = 19,  $p$ -value <  $2.2 \times 10^{-16}$ ) and  $R=0.961$  of C ( $t=15.254$ , d.f. = 19,  $p$ -value =  $4.094 \times 10^{-12}$ ).

### (h) Bayesian mixing model

FRUITS (v. 3.0 beta) was used to generate the Bayesian mixing model (available at <http://sourceforge.net/projects/fruits/>). The model was adapted from Model 2 reported by Soncin *et al.* [40], a concentration-dependent model that uses  $\delta^{15}\text{N}$  values of source (Phe) and trophic (Glx) amino acids to estimate protein contribution. The  $\delta^{15}\text{N}$  values of Phe and Glx of the human individuals from El Collado (i.e. *targets*) were analysed against the two fractions 'Phe' and 'WholeN' of animal products and marine and freshwater fish, respectively. Human  $\delta^{15}\text{N}$  values of phenylalanine are directly linked, with negligible isotopic fractionation, to the  $\delta^{15}\text{N}$  values of phenylalanine of the protein sources, *fraction* 'Phe', which corresponds to the source  $\delta^{15}\text{N}$  values of phenylalanine. While  $\delta^{15}\text{N}$  values of glutamic acid are the results of the transamination reactions of the nitrogen metabolic pool; *fraction* 'Whole N', which corresponds to the source bulk  $\delta^{15}\text{N}$  values [53]. The *offsets* from diet to consumer were obtained from multiple feeding experiments studies reported by Soncin *et al.* [40] and these are:  $\Delta^{15}\text{N}_{\text{Phe-Phe}} = +0.1 \pm 0.2$  ‰ and  $\Delta^{15}\text{N}_{\text{Glx-wholeN}} = +9.7 \pm 2.5$  ‰. As sources, we used terrestrial herbivores from El Collado representing 'animal products' and marine fish from El Collado and Cueva de Nerja (electronic supplementary material, table S1) and freshwater fish from Naito *et al.* [39] representing 'fish'. The concentrations of the two fractions 'Phe' and 'WholeN' were derived from the USDA National Nutrient

Database for Standard Reference (available at <https://fdc.nal.usda.gov/>) and expressed as dry weight (%). The items used from the USDA database are the same reported by Soncin *et al.* [40]. The associated uncertainties of all the reported measurements are standard errors of the mean. Model estimates are reported in electronic supplementary material, table S3.

### 3. Results and discussion

The criteria for assessing the suitability of collagen followed those reported in [40]. In total, collagen of sufficient quality for bulk elemental analysis-isotope ratio mass spectrometry (EA-IRMS), and determination of both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of at least 13 amino acids by gas chromatography combustion-IRMS (GC-C-IRMS), was obtained from all 11 human individuals, all 4 terrestrial fauna, but only 6 out of 16 of the marine fish bones (electronic supplementary material, table S1). When compared to bulk stable isotope data [29] the differences are typically  $\pm 1\%$ , although here we provide additional stable isotope data on four additional individuals (COLL 8, 10, 11 and 14) whereas three individuals (COLL 1, 2 and 9) were not available for further analysis. This dataset was compared with previously published bulk and single compound stable isotope data from freshwater fish recovered from the Epipalaeolithic rockshelter of Pont d'Ambon located in Western France dating from between (13 000 to 9500 cal BP [39]).

#### (a) Bulk collagen results

The bulk stable isotope ratios obtained for the faunal skeletal material from El Collado and Cueva de Nerja (electronic supplementary material, table S1, figure 2a) confirms previous studies [35] that show a lack of clear distinction in  $\delta^{15}\text{N}_{\text{coll}}$  between marine and terrestrial fauna; although the mean values for the terrestrial species are lower than the marine, the ranges partially overlap (figure 2a). The  $\delta^{13}\text{C}_{\text{coll}}$  clearly discriminates marine from terrestrial and freshwater organisms but not terrestrial from freshwater fauna. One of the *Bos primigenius* samples had a  $\delta^{13}\text{C}_{\text{coll}}$  value of  $-15.71\%$ , falling outside the range for the consumption of terrestrial  $\text{C}_3$  plants suggesting consumption of wild  $\text{C}_4$  plants or marine macroalgae, as has been previously suggested for wild fauna foraging along coastal regions [56,57]. Overall, the variability in the bulk isotope values of both marine and terrestrial fauna introduces more uncertainty when determining the contribution of these sources to human diets as is apparent in figure 2a, which also includes freshwater fish from the Pont d'Ambon. The bulk collagen values of the humans from El Collado measured in this study range from  $-19.2\%$  to  $-17.4\%$  for  $\delta^{13}\text{C}_{\text{coll}}$  and  $8.86\%$  to  $13.44\%$  for  $\delta^{15}\text{N}_{\text{coll}}$  (electronic supplementary material, table S1) and are in-line with previous measurements of this assemblage [29].

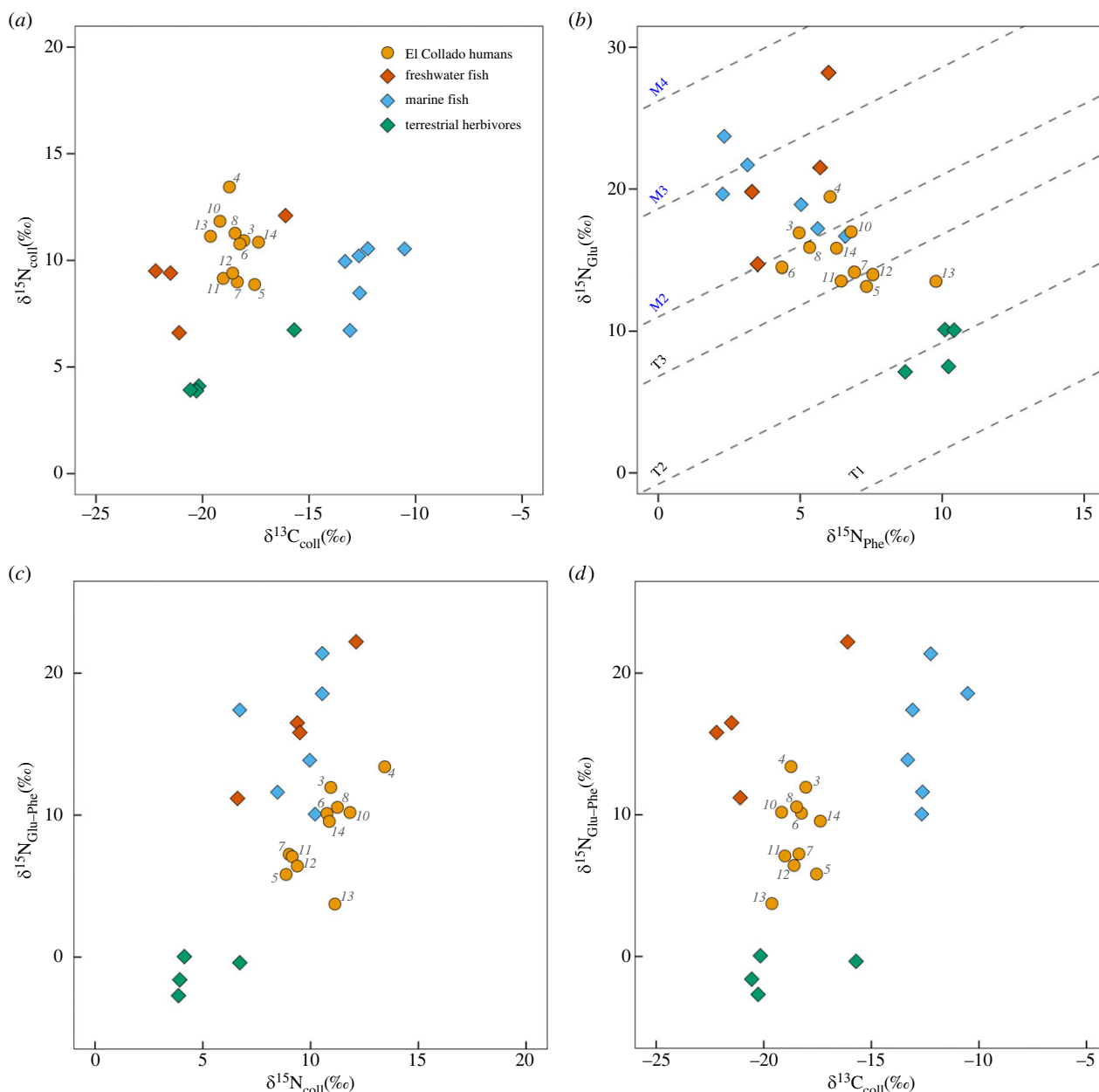
#### (b) Estimation of trophic position using compound-specific isotope analysis of amino acids (CSIA-AA)

Compound-specific isotope analysis of amino acids is finding increased application in ecology to estimate a consumer trophic position [38,58]. The benefit of this approach is that it allows simultaneous estimation of the baseline  $\delta^{15}\text{N}$  value by considering the values of source amino acids, as well as those fractionated through trophic transfer (i.e. trophic amino acids). The approach has been applied to archaeological

human remains, for example, to investigate the degree of aquatic food consumption in hunter-gatherers in central France and coastal Japan [39,58]. While the precise estimation of trophic position and dietary quantification using this approach is complicated by variability in the values of the source and trophic amino acids in primary producers at the base of the food web ( $\beta$  value) [38,58] and the trophic enrichment factor (TEF), further exacerbated for human with diets consisting of mixed terrestrial and marine foods [40], such comparisons nonetheless provide a useful indicator. A comparison of the  $\delta^{15}\text{N}$  values of glutamic acid (Glu; a trophic amino acid derived from both glutamic acid and glutamine residues in bone collagen) and Phe (a source amino acid) is presented in figure 2 with the bulk collagen data ( $\delta^{13}\text{C}_{\text{coll}}$  and  $\delta^{15}\text{N}_{\text{coll}}$ ). The Glu-Phe  $\Delta^{15}\text{N}$  values for the terrestrial herbivores (figure 2b) range from 0–3‰ and are in line with other measurements made on southern European prehistoric and historic terrestrial animals [39,40]. Interestingly six out of the 11 human individuals have  $\Delta^{15}\text{N}_{\text{Glu-Phe}}$  values placing them at one trophic position above these terrestrial animals (figure 2b). Instead, these values are within the range of contemporary marine fish measured in this study and marine and freshwater fish measured elsewhere [39,40].

Considering the new data presented here, it is evident that aquatic products were much more prominent in the diet than originally postulated using bulk isotope analysis [29]. This is explained by only a moderate correlation (Pearson's;  $R = 0.62$ ; 0.30 to 0.82 (95% confidence),  $p$ -value = 0.0008) between bulk  $\delta^{15}\text{N}_{\text{coll}}$  and  $\Delta^{15}\text{N}_{\text{Glu-Phe}}$  (figure 2c), if all the human and faunal samples are considered. The  $\Delta^{15}\text{N}_{\text{Glu-Phe}}$  values result in much greater discrimination between aquatic and terrestrial fauna compared to  $\delta^{15}\text{N}_{\text{coll}}$ , as noted by Soncin *et al.* [40]. Therefore, even high trophic level Mediterranean fish have  $\delta^{15}\text{N}_{\text{coll}}$  that cannot be easily discriminated from terrestrial fauna due to their relatively low  $\delta^{15}\text{N}$  values of source amino acids (e.g. Phe) (figure 2b). This observation also seems to be true of freshwater fish from the Epipalaeolithic site of Pont d'Ambon in Southwestern France [39] but could indeed vary spatially and temporally due to differences in nitrogen assimilation by primary producers. However, Garcia-Guixé *et al.* 2006 [29] also drew on the bulk collagen  $\delta^{13}\text{C}$  values to make their interpretation. By plotting  $\Delta^{15}\text{N}_{\text{Glu-Phe}}$  against  $\delta^{13}\text{C}_{\text{coll}}$  (figure 2d), the marine fauna is clearly enriched in  $\delta^{13}\text{C}$  compared to the humans despite the overlap in the range of  $\Delta^{15}\text{N}_{\text{Glu-Phe}}$  values. One reason for this is that El Collado fish assemblages were dominated by gilthead bream, *Sparus aurata* [17] a euryhaline marine teleost [59] that is likely to have resided in nearby brackish lagoons or estuaries adjacent to the site. Unfortunately, no sea bream was available for analysis from El Collado and the single individual measured from Cueva de Nerja may have resided in more saline, open sea environments given the site's location.

To investigate further, we examined the  $\delta^{13}\text{C}$  of amino acids from the El Collado humans and faunal samples. Unfortunately, only 8 of the human samples and the marine fish samples passed the stringent quality control criteria (see Materials and Methods) implemented by Soncin *et al.* [40]. It has been shown that the  $\delta^{13}\text{C}_{\text{AA}}$  values of essential amino acids such as valine, phenylalanine and leucine can adequately distinguish marine, freshwater and terrestrial consumers [60,61]. In figure 3, we have plotted the  $\delta^{13}\text{C}$  of valine against phenylalanine and compared them with global references for  $\text{C}_3$ , terrestrial and marine consumers reported by



**Figure 2.** Plot of *a*) Bulk collagen  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of humans and fauna from El Collado, Cueva de Nerja compared with freshwater fish from Pont d'Ambon [39]; *b*)  $\delta^{15}\text{N}$  of glutamic acid against the  $\delta^{15}\text{N}$  of phenylalanine from collagen; the difference between  $\delta^{15}\text{N}_{\text{Glu}}$  and  $\delta^{15}\text{N}_{\text{Phe}}$  values from collagen against *c*) bulk collagen  $\delta^{15}\text{N}$  and *d*) bulk collagen  $\delta^{13}\text{C}$ . The trophic position lines (T = terrestrial TP, M = marine TP) shown in B are from Naito [54] for reference only (see [55]).

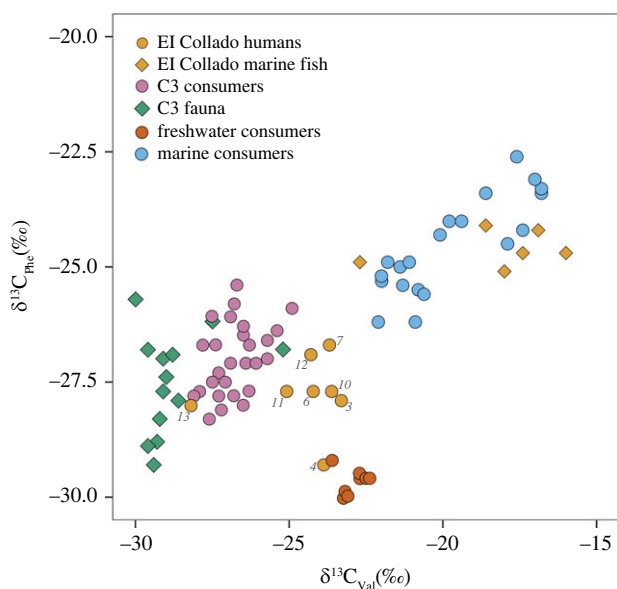
Shulting [52]. These comparative data are drawn from Europe and East Asia as none are available for the Mediterranean region. Firstly, the marine fish from the Mediterranean analysed here plot broadly within the range of marine protein consumers from elsewhere, which include marine species and coastal hunter-gatherers from Japan and Greenland (electronic supplementary material, table S2). Secondly, the analysis of humans from El Collado  $\delta^{13}\text{C}_{\text{AA}}$  broadly corresponds with the  $\delta^{15}\text{N}_{\text{AA}}$  identifying Coll 4 as a high trophic level 'aquatic' consumer and Coll 13 as the lowest trophic level 'terrestrial' consumer. However, with the  $\delta^{13}\text{C}_{\text{AA}}$  analysis, we were able to confirm that freshwater or brackish fish was an important dietary source at El Collado for most of the individuals, as evident in figure 3.

### (c) Multivariate analysis

Consideration of a greater range of amino acid stable isotope measurements can provide further insights into the human

diet at El Collado than that obtained using only the proxies considered above. To do this, we conducted principal components analysis (PCA) [62] on a wider range of amino acids to distinguish the different taxonomic groups and individuals and second by using a Bayesian mixing model to quantify individual human diets based on prior assumptions of amino acid metabolism [40]. Given that the  $\delta^{13}\text{C}$  measurements were not available for terrestrial fauna and are probably an inappropriate dietary endpoint, as discussed above, they are omitted from this analysis.

The PCA analysis conducted on all the available amino acid  $\delta^{15}\text{N}$  measurements (12 in total; electronic supplementary material, figure 4) shows that the specimens cluster according to their taxonomic groupings (humans, marine fauna, terrestrial herbivores). Both PCA 1 (61.3% of the variation) and PCA 2 (21.3% of the variation) effectively distinguish these different groups. The humans plot intermediary between marine and terrestrial baselines on component one, implying that at least some of the amino



**Figure 3.** Plot of  $\delta^{13}\text{C}$  of valine against the  $\delta^{13}\text{C}$  of phenylalanine from collagen extracted from marine fish and humans from El Collado compared with previously published values [52].

acid nitrogen was obtained from a marine-brackish source. As may be expected for higher trophic level marine organisms, most amino acids are enriched in  $^{15}\text{N}$  in the Mediterranean fish samples. Conversely, as noted in §3b, phenylalanine is enriched in  $^{15}\text{N}$  in the terrestrial herbivores compared to the marine fish and provides an effective means of discrimination in this context (figure 4c). Threonine is also relatively enriched in the herbivores compared to humans and marine fish. Threonine is atypical in that with increasing trophic level its  $\delta^{15}\text{N}$  values will decrease due to preferential enzymatic catabolism of  $^{15}\text{N}$ -Thr to alpha-ketobutyrate and ammonia leading to  $^{15}\text{N}$  depletion relative to the dietary source, with the magnitude of the effect influenced by the amount of dietary protein and/or gluconeogenesis [63]. The El Collado human  $\delta^{15}\text{N}_{\text{thr}}$  values for the most extreme marine consumers are within the range of the fish (electronic supplementary material, table S1) but the degree to which this is due to protein intake and dietary quality is difficult to assess.

Interestingly, PC2 distinguishes humans from both terrestrial and marine fauna (figure 3) and is correlated with the two biosynthetically related glucogenic amino acids, serine and glycine, and to a lesser degree lysine, proline and hydroxyproline. The relative enrichment in  $\delta^{15}\text{N}_{\text{gly}}$  from the human samples compared to the marine and terrestrial faunal samples are shown in figure 3c. One explanation is that for humans, the nitrogen atom in glycine (and serine) was preferentially obtained through extensive transamination and therefore derived to a greater extent from the metabolic nitrogen pool [64]. Whereas a greater proportion of glycine is directly routed from dietary sources in the terrestrial and marine fauna. The metabolic and dietary conditions to explain this effect are unclear and potentially multifactorial [63], one of which is the amount of protein in the diet. As can be seen in figure 4a, the correlation between different human individuals and component 2 is variable which might be related to the degree of routing versus synthesis of glycine or related to the  $\delta^{15}\text{N}$  values of nitrogen in the metabolic pool. The relative enrichment in glycine in humans partly explains their higher than predicted  $\delta^{15}\text{N}_{\text{coll}}$  values when the Glu-Phe proxy is considered (figure 2c), as glycine is the most dominant amino acid in bone collagen.

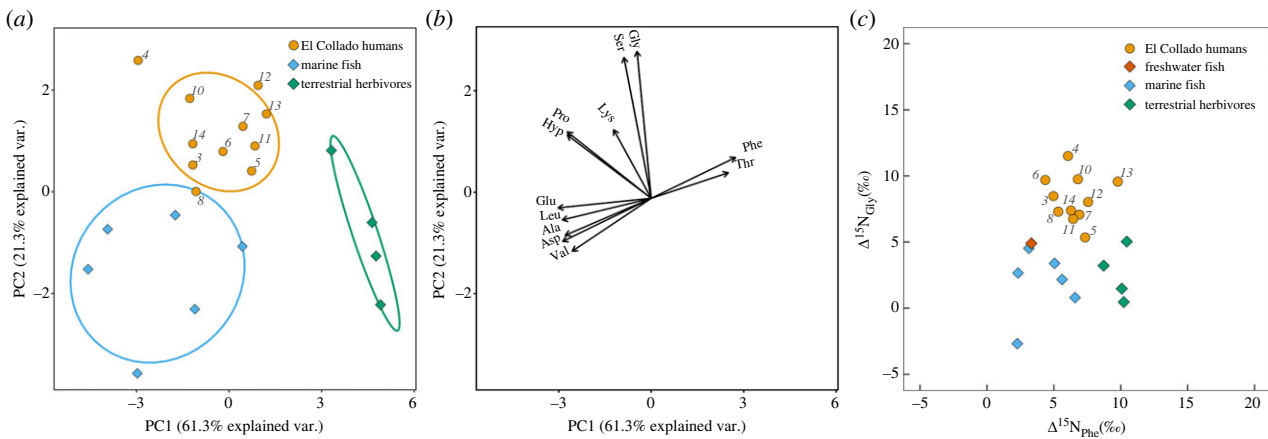
#### (d) Quantifying protein consumption using a mixing model

To quantitatively contextualize and evaluate dietary variability at El Collado, we developed a mixing model adapted from Soncin *et al.* [40]. This model (figure 5) considers nitrogen-stable isotope values from one source amino acid, phenylalanine and one trophic amino acid, glutamic acid (derived from glutamic acid and glutamine residues). The data are obtained directly from terrestrial fauna and aquatic organisms, the latter comprising marine fish from El Collado and Cueva de Nerja, and freshwater fish previously published from Pont d'Ambon [39]. The advantage of this model is that it excludes any variability in the  $\delta^{13}\text{C}$  between marine and freshwater fish. This is particularly relevant here considering we know from the  $\delta^{13}\text{CAA}$  measurements that freshwater or brackish fish were likely to have been an important source of dietary protein. Using this model (figure 5), we estimate that the aquatic protein contribution to total dietary protein could have been as high as 80% for some of the individuals at El Collado (electronic supplementary material, table S3). The estimates provided by the mixing model show relatively high uncertainty compared to previous studies [40] since the model only relies on only  $\delta^{15}\text{N}$  values of just two amino acids. Nevertheless, the compound-specific approach used here provides greater accuracy, as a result of the limited number of assumptions made, and overall brings dietary variability across the assemblage into much sharper relief compared to previous studies that considered only the bulk stable isotope values (figure 2a) [29].

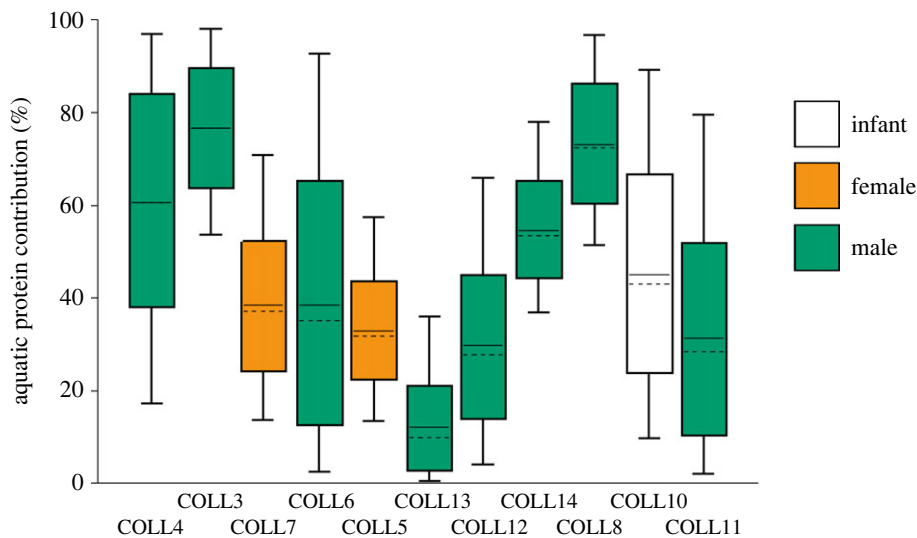
#### (e) Dietary variability at El Collado

Using either multivariate analysis of the amino acids nitrogen values or by observing the different proxies, there seems to be high variability in consumption of marine and terrestrial protein across the El Collado burial ground, from diets clearly dominated by aquatic foods to those where it was conceivably absent. The collagen signal represents the averaging of diet over many years prior to death although weighted to periods of rapid bone growth during adolescence [65]. The results, therefore, reflect enduring practices related to access to foodstuffs or dietary preferences. Most significantly, the dietary reconstruction using CSIA on El Collado calls for the re-evaluation of the importance of aquatic resources to pre-agricultural diets in the Western Mediterranean Basin. Faunal and bulk collagen stable isotope analysis picture the role of marine resources as occasional and sporadic food supplements to subsistence systems dominated by terrestrial mammals and plants [15–18,21,23,25]. Our study challenges this conventional view by showing that at least in shelf waters of the north-western Mediterranean, brackish coastal environments could have supported subsistence systems strongly dependent on fishing and shellfish exploitation, notably in times of increased resource pressure on land and rapid human population growth of the Late Pleistocene and Early Holocene [66,67].

It is tempting to link this variability to the different tasks performed by individuals within the community during their lifetime and is less consistent with the hypothesis that foods were shared equally across the group, as sometimes envisaged in models of egalitarian societies which have been often drawing on contemporary or historical accounts



**Figure 4.** Principal component analysis of  $\delta^{15}\text{N}$  values of 12 amino acids measured in human, terrestrial animal and marine fish bone collagen (a). To facilitate the visualization of the data, the vectors have been plotted in a separate graph (b). The  $\delta^{15}\text{N}$  values of phenylalanine against glycine are plotted in figure 4c.



**Figure 5.** Estimated aquatic protein contribution in the individuals from El Collado using a Bayesian mixing model applied to  $\delta^{15}\text{N}_{\text{Phe}}$  and  $\delta^{15}\text{N}_{\text{Glu}}$  values. Individuals are organized chronologically from early to late phases of the Mesolithic sequence (9475–8408 cal BP) following [41]. Boxes represent a 68% credible interval (corresponding to the 16th and 84th percentiles) while the whiskers represent a 95% credible interval (corresponding to the 2.5th and 97.5th percentiles). The horizontal continuous line represents the mean while the horizontal discontinuous line represents the median (50th percentile).

of hunter–gatherers [53,68]. While such assumptions are highly questionable anyway [69], we also need to consider that El Collado burial ground represents individuals buried over a long period of time, perhaps up to a millennium and at the very least seven centuries [41], providing scope for considerable change in food procurement strategies. There are no clear patterns between diet and burial phase (figure 5; electronic supplementary material, table S1); there is considerable dietary variation both within the earliest group of burials (Coll 4, 3 and 7) and the later ones, perhaps pointing to dynamic food procurement strategies even over relatively short periods. The degree of residential mobility among the group is also unclear but perhaps the cemetery served more than one community primarily located in different areas of the landscape (e.g. coastal lagoon, littoral, forest). Similarly, there are not enough data to suggest sex-based dietary differences, with only two females and perhaps an additional maternal signal partially represented through the diet of the 9-month-old infant (Coll 10), who was probably breastfeeding at the time of death [70,71].

## 4. Conclusion

The overall aim of this study was to clarify the relative contribution of aquatic resources to the diets of the Mesolithic coastal population at El Collado. Using a CSIA approach applied to collagen amino acids we have highlighted lagoonal aquatic resources, such as demersal fish, were a major source of dietary protein to 4 out of the 11 individuals investigated, and this component was likely to have been non-negligible in all but two individuals. These new findings reflect a strong coastal-oriented economy which included individuals who were routinely involved in the capture and processing of fish and presumably shellfish, and who may have possessed the knowledge and the technology to occasionally pursue fishing in open marine environments in other locations. We argue against the notion that aquatic foods served only as a fall-back or seasonal resource, although this may have been the case in some instances. For some individuals, the contribution of marine resources to diet approaches the level observed among Mesolithic hunter–gatherers of the Atlantic coast, implying that any differences in productivity or inter-tidal harvestable



biomass did not have a major impact on diet, although specific fishing and harvesting practices might have varied between regions. This study rekindles a longstanding debate regarding the origin and changing nature of coastal subsistence strategies in the Mediterranean. Our data show that the region was, at least, capable of supporting 'marine' adapted coastal groups in the Mesolithic period with similar diets to those described for the European Atlantic façade [72–74].

The study also highlights the advantage of deploying compound-specific isotope analysis in Mediterranean contexts, especially when the bulk collagen  $\delta^{15}\text{N}$  fails to adequately discriminate between aquatic and terrestrial consumers. By using other proxies from single amino acids, we were able to circumvent this problem, identify additional dietary sources and more effectively quantify the degree of aquatic intake. Missing sources, such as terrestrial plants and shellfish, prevent accurate quantification, but by relying on source amino acids that only minimally fractionate from through the food chain, this issue is partially mitigated. The data presented here will also be of importance when considering amino acid isotope measurements of humans from the earliest agricultural societies in the Western Mediterranean, to assess the degree of dietary change associated with the introduction of domesticated plants and animals.

**Data accessibility.** All data generated by the study are present in the paper and reported in the electronic supplementary material [75].

**Authors' contributions.** M.F.-C.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation,

visualization, writing—original draft, writing—review and editing; S.S.: data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing; H.M.T.: data curation, formal analysis, writing—review and editing; M.v.T.: data curation, formal analysis, writing—review and editing; J.F.G.: investigation, writing—review and editing; A.C.C.: investigation, writing—review and editing; O.E.C.: conceptualization, data curation, formal analysis, supervision, validation, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

**Conflict of interest declaration.** We declare we have no competing interests.

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