

Stable isotope evidence of the diet of the Neolithic population in Slovenia – a case study: Ajdovska jama

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ABSTRACT – *The aim of this research was to determine the nutrition habits of Neolithic people living in Slovenia between 4000 BC and 3400 BC. The specific isotopic composition of different types of food is reflected in the isotopic composition of the tissues of the consumer. Therefore, by measuring the isotopic composition in the tissues we can draw conclusions about the nutritional habits of the consumer. We analysed the remains of human bones taken from Ajdovska jama and determined the stable isotopic composition of carbon and nitrogen in the bone collagen. Our results indicate that the diet consisted primarily of herbivores, most probably domestic animals.*

IZVLEČEK – *Namen našega dela je ugotoviti prehranjevalne navade neolitskega človeka na naših tleh iz petega in četrtega tisočletja BC. Izotopska sestava ogljika in dušika v hrani je različna in se odraža v izotopski sestavi tkiv uživalca, zato lahko na podlagi meritev izotopske sestave sklepamo o njegovem prehranjevanju. Analizirali smo vzorce kostnih ostankov iz Ajdovske jame in določili izotopsko sestavo ogljika in dušika v kostnem kolagenu. Rezultati meritev kažejo, da so pretežno prehrano naših prednikov predstavljale rastlinojede, v glavnem domače živali.*

KEY WORDS – *Ajdovska Jama; Neolithic diet; stable isotopic composition of carbon and nitrogen*

INTRODUCTION

Since its introduction in 1977, stable isotope analysis of bone collagen has become a very valuable tool for determining prehistoric human and animal diets (DeNiro and Epstein 1978; Tauber 1981; Schwartcz and Schoeninger 1991; Lubell et al. 1994; Schulz 1998). The inorganic and organic chemical constituents of bone provide a record of long-term dietary intake. Elements and amino acids liberated by the digestion of food are incorporated into bone minerals, collagen and non-collagenous bone proteins throughout a vertebrate's lifetime. Dietary information is thus recorded by carbon and nitrogen isotope ratios in bone collagen and by carbon isotope ratios in the carbonate component of the inorganic portion of bone minerals (bioapatite) and teeth mineral (Krueger and Sullivan 1984). The reconstruction of individuals' diets using isotopic methods has been limited to the analysis of collagen preserved in bone, because bioapatite is more difficult to deal with due to problems of diagenesis (Schoeninger and DeNiro 1982).

Carbon isotopes are fractionated by natural processes such as the photosynthetic assimilation of CO₂ and its adsorption in water. Carbon fractionation is affected by the type of metabolism used by a plant to fix CO₂ and differs in the marine and terrestrial foods chain and may therefore be used to elucidate questions on the origin of naturally occurring carbon compounds. Due to kinetic isotope effects, terrestrial plants that follow normal Calvin (C₃) photosynthesis are depleted in the heavy carbon isotopes, as is shown in a change in δ¹³C values from -6 to -8‰ in atmospheric CO₂ to -24 to -32‰ in terrestrial plants. Terrestrial animals and human feeding on such plants show a similar ¹³C content, although with a slight shift towards higher δ¹³C values. The absorption of CO₂ in water and the subsequent formation of bicarbonate are governed by kinetic isotope effects and by thermodynamic equilibrium processes, which lead to an enrichment in heavy carbon isotopes to δ¹³C values closed to 0‰. When marine bicarbonate is assimilated during pho-

tosynthesis in submerged plants, reaction kinetics again result in depletion in the heavy isotopes, in this case to $\delta^{13}\text{C}$ values about -10 to -18‰ , and this fractionation is also reflected in marine animals and human whose food was based mostly on marine protein. The simple isotopic separation between terrestrial and marine plants and animals is partially obscured if the C_4 photosynthetic cycle has dominated in terrestrial plants. The isotopic composition of these plants ranged between -10 to -16‰ . The most important cultivated C_4 plants were maize, sugar cane and millet. However, in our study, which concerns temperate Europe, the influence of C_4 plants can be excluded.

Little is known about the nitrogen isotopic composition of different types of food. It has been suggested that plants that can fix molecular nitrogen (due to the presence of symbiotic bacteria) have characteristically lower $^{15}\text{N}/^{14}\text{N}$ ratios than those which must assimilate other forms of inorganic nitrogen, such as ammonia or nitrate (Delwiche *et al.* 1979). The differences between the $\delta^{15}\text{N}$ values of the two types of plant appear to vary depending on the location in which they grew and the time of year at which they were collected (DeNiro and Epstein 1981). The basis for the geographical and temporal variability of plant $\delta^{15}\text{N}$ values must be resolved before a dietary analysis based on the isotopic ratios of animal nitrogen can be exploited to its full potential. It may also be possible to use the nitrogen isotopic method of dietary analysis to determine the relative amounts of terrestrial and aquatic food sources eaten by animals living in shore environments. Stable nitrogen isotopes, ^{15}N , are also enriched in

marine systems relative to terrestrial systems, but for such studies the degree of trophic level fractionation is more important. Enrichment of ^{15}N through the trophic network is widely recognised among most animals, including invertebrates and vertebrates, leading to a value of $3.4 \pm 1.1\text{‰}$ (Minagawa and Wada 1984; Wada *et al.* 1987). These facts suggest that the isotopic composition of organisms provides basic information not only about their food source, but also the trophic level. Most marine fish that are eaten by humans are carnivores, and marine food chains are relatively longer than terrestrial chains, therefore ^{15}N contents are relatively high. The is true of lake fish, so that humans consuming a substantial proportion of fish and/or mammals will have higher stable nitrogen value than is possible to attain in a purely terrestrial system (Schoeninger *et al.* 1983; Katzenberg 1989).

Two important issues must also be considered. First, stable isotope results on human bones provide insights into the amount of protein an individual has consumed over approximately the last ten years of their lifetime (Chisholm *et al.* 1983, Schwarcz and Schoeninger 1991). And the second, the assumption that the bone collagen isotope ratio has not been modified by post-mortem processes. It was shown that the material isolated from prehistoric bones, with C/N ratios between 2.9 and 3.6 have not undergone diagenetic alteration (DeNiro 1985). It is possible they have, but previous studies suggest that such diagenetic shifts must be small enough for identification of the basic feeding behaviour of the individuals from their bone collagen isotope ratios to be possible.



Fig. 1. Ajdovska jama near Nemška vas is located in the south-eastern foot-hills of the Krško highlands.

Fig. 2. Human skeletons were discovered in the left corridor and in the central hall of the cave.



The aim of this paper is to determine the nutritional habits of Neolithic people living in Slovenia between around 4000 BC and 3400 BC using isotopic methods. The isotopic composition of different plant and animal remains in association with human skeletons were also determined in order to be able to define more precisely the roles of plants and animal protein in the diet of the humans living at the time.

MATERIALS AND METHODS

The human bone samples from Ajdovska jama near Nemška vas, also known as Kartušova jama located in the south-eastern foot-hills of the Krško highlands were collected in this study (Fig. 1). Human skeletons were discovered in the left corridor and in the central hall (Fig. 2). Anthropologists managed to identify 29 individuals, namely 13 adults (6 males and 7 females) and 16 infants. Different faunal species associated with the burials were also found. The most represented species were domesticated mammals, such as cattle, sheep, pigs, and plants – mostly wheat, which implies some domestic activity during both periods. Also, some remains of wild animals were found: brown bear, deer, field hare. The floral and faunal remains and the human bone samples were then transferred to labelled, polythene bags which were sealed until the start of pre-treatment. The AMS ^{14}C analysis was used to date the burial remains in Ajdovska jama. The results indicate that the samples are from two different periods *ca* 5300 and 6000 yrs BP.

Sample preparation

Since the accuracy of measurements mainly depends on the variability inherent in the collagen extraction and measurement techniques, we have compared

two methods that have been developed for collagen extraction and purification. Four samples were selected for this test. The bone samples were cleaned in cold, distillate water in an ultrasonic bath to remove soil contaminants, and then oven-dried at 50°C to constant weight. The samples were ground in a mill to ~1 mm fine powder and subdivided into two portions. The first extraction method was that described by Longin (1971). Approximately 1 g of bone powder was weighed into a 250 ml beaker and 150 ml of 1M hydrochloric acid (HCl) added to remove the acid soluble inorganic portions of the bone, any acid soluble protein and peptide fragments, and free amino acids. The acid also breaks down some of the hydrogen bonds of collagen, so that it becomes soluble in hot water. The pre-treatment time must be short (< 20 min), otherwise the proteinic chain is hydrolysed and the collagen becomes soluble in hot water and is then lost. The acid solution is then discarded by filtration through a glass microfibre filter and well washed with distillate water. The remaining acid insoluble material, which includes undenatured, and insoluble collagen, is extracted under reflux for 10 hours in a hot water (90°C) of pH = 3. The heating serves to denature and partially hydrolyse the intact collagen, making it soluble, while the acidic pH 3 avoids dissolving any non-acid-soluble contaminants. The solution is then filtered through an 8 µm polyethylene filter to remove insoluble residues, and the collagen isolated by freeze-drying the filtrate.

Method 2 is the modification of the Longin method suggested by Richards and Mellars (1998). In this case, the inorganic portion is removed by extraction with 0.5 M HCl solution. The samples were kept at 4–5°C overnight. Powdered samples collected on glass microfibre filters were washed twice with distillate water. Then the residue was placed in a sealed

tube (under reflux) in a pH 3 HCl solution, and gelatinised for 48 hours at 75°C. The solution is then decanted and filtered through an 8 µm polyethylene filter and freeze-dried. This more gentle treatment is intended to reduce the collagen loss as compared to method 1.

No measurable effects on the determining of $\delta^{13}\text{C}$, and only a small effect on the $\delta^{15}\text{N}$ signal were obtained using these two methods. We conclude that the best collagen extraction technique is the second method proposed by Richards and Mellars (1998). Method 1 gives essentially the same results, but the collagen recovery may be lower.

Other researchers have included a sodium hydroxide wash in their preparation sequence (DeNiro and Epstein 1981; Pate 1995). The sample was treated before the hot water extraction step with 0.5% sodium hydroxide (NaOH) for ~20 h to remove base soluble contaminants such as humic acids. The results from other studies show that this pre-treatment has little effect on measured $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, and because of the apparently reduced yields (Chrisholm et al. 1983; Bonsall et al. 1997) we decided not to use this method.

Isotopic analysis

The isotopic composition of collagen was then determined using a Europe Scientific 20/20 continuous flow mass spectrometer with ANCA-SL solid-liquid preparation module. The technique involves the coupling of a preparation system employing the Dumas principle with a stable isotope mass spectrometer detector. This allows measurements of not only total nitrogen and carbon in a sample, but also their ^{15}N and ^{13}C levels. The "collagen" solid was placed in tin cups and dropped sequentially into a combustion tube as a pulse of oxygen was injected. After various reduction reaction and chemical trapping, carried out in a helium carrier which transports released gases, the gas chromatography (GC) column separates N_2 and CO_2 from trace impurities before analysis by the IRMS (Isotope Ratio Mass Spectrometer). The samples are analysed in batches that include known references (working standards). References calibrate isotopic abundance and elemental composition measurements and allow correction for drift. As a working standard, pure collagen was used which was calibrated vs. reference materials (IAEA-CH-7 polyethylene and NBS22 oil for carbon; IAEA-N-1 and IAEA-NO-3 for nitrogen).

Following standard procedure, the isotopic ratios are expressed in δ -notation in parts per mil (‰):

$$\delta^*X = \left[\frac{(*X/X)_{\text{sample}}}{(*X/X)_{\text{standard}}} - 1 \right] \times 1000$$

For carbon, $*X/X$ is $^{13}\text{C}/^{12}\text{C}$ and the standard is the V-PDB carbonate, while for nitrogen $*X/X$ is $^{15}\text{N}/^{14}\text{N}$ and the standard is atmospheric (air) nitrogen. The measurement uncertainties on the $\delta^{13}\text{C}$ values were $\pm 0.2\text{‰}$, and $\pm 0.3\text{‰}$ on the $\delta^{15}\text{N}$ values.

The reliability of stable isotopic analyses of collagen depends somewhat on the degree of preservation of collagen, which can be estimated from its C/N ration. Well-preserved collagen should display a C/N ratio between 2.9–3.6 (DeNiro 1985). As mentioned above, the continuous-flow mass spectrometer allows the determination of isotopic abundance and a sample composition (N content, C/N ratio) at the same time. Therefore, in our collagen samples the C/N ratios were also determined. The results are collected in table 1 and 2. Almost all samples fell within the prescribed range, suggesting that the collagen samples are in general well-preserved. The exceptions are the two samples marked with * in table 1. These sample were eliminated from our study because their $\delta^{13}\text{C}$, or more probably, $\delta^{15}\text{N}$ values might have shifted substantially and thus their use in dietary reconstruction might lead to erroneous conclusions.

RESULTS AND DISCUSSION

The isotopic composition of food sources in diet

As a part of the present study, samples of various foods (cultivated vegetables, meat from domestic and wild animals) in association with human skeletons were also analysed for their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. The results are collected in table 1 and shown in figure 3. These results can serve as a base against which the stable isotopic measurements on human bone collagen can be compared to indicate diet, when allowance is made for the enrichment of ca 5‰ in $\delta^{13}\text{C}$ and 3‰ in $\delta^{15}\text{N}$ between consumers and the food consumed (van der Merwe and Vogel 1978; Schoeninger 1985). All cereal remains are C_3 plants with an average $\delta^{13}\text{C}$ value of $-24.7 \pm 1.5\text{‰}$ and $\delta^{15}\text{N}$ value of $+3.3 \pm 1\text{‰}$. The difference in the isotopic composition in plants was also observed. Leguminous plants have $\delta^{15}\text{N}$ values of $+1.9\text{‰}$, while the non-legumes, in our case wheat, have $\delta^{15}\text{N}$

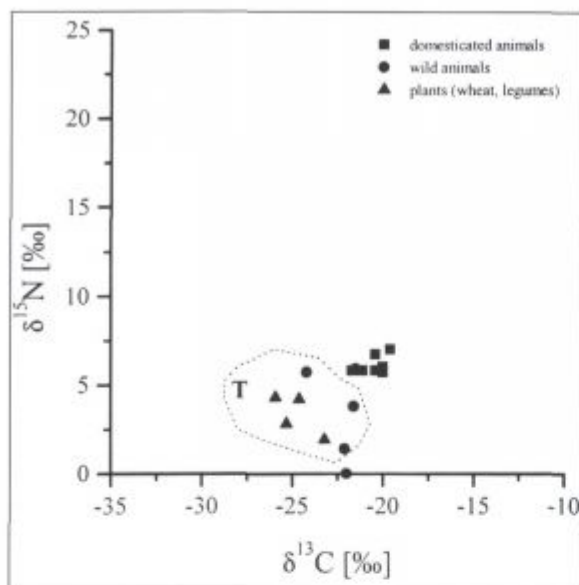


Fig. 3. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for foods available in Neolithic and terrestrial (T) herbivores established by Schwarcz (1991).

values of $+3.8 \pm 0.4\text{‰}$. Also, the results obtained from animal bones show that their diets favoured C_3 plants. The clear distinction between domestic and wild animals was observed in the $\delta^{15}\text{N}$ values. Samples from terrestrial wild herbivores have $\delta^{15}\text{N}$ values in the range of 0 – 6‰ (average = 3.7‰), while seven samples from domestic animals analysed have $\delta^{15}\text{N}$ values between 5 – 7‰ (average = 6.1‰). These results are in good agreement with the two studies concerning analyses of wild animals performed by Schwarcz (1991), and domesticated animals available today in temperate Europe (Bonsall *et al.* 1997). It is still not clear why the $\delta^{15}\text{N}$ values of wild and domesticated herbivores differ so markedly. Most probably the difference is connected with feeding patterns. Domesticated animals could be fed foodstuffs that were not available to wild herbivores. It is conceivable that animals kept by farmers ingested some cultivated vegetables (the $\delta^{15}\text{N}$ values are -3‰ higher than that observed in plants – wheat, legumes) and also a certain amount of human food refuse. Our results for prehistoric domesticated animals correspond well with those obtained from modern livestock, suggesting that animals had similar foodstuffs and should be regarded as having more omnivorous diets than their wild counterparts.

Palaeodietary reconstruction

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of collagen extracted from human bones from two different periods 5300 and 6000 BP are collected in table 2 and graphically presented in

Sample	Age (yr BP)	$\delta^{13}\text{C}$ [‰]	$\delta^{15}\text{N}$ [‰]	C/N
ANIMALS				
domestic cattle	5300	-21.1	+5.8	3.4
domestic sheep	5300	-19.6	+7.0	3.2
deer *	5300	-24.3	+6.3	6.7 *
domestic cattle	?	-20.4	+6.7	3.4
brown bear	?	-22.1	+1.4	3.2
deer *	?	-24.2	+5.7	6.5 *
domestic cattle	6000	-21.7	+5.8	3.4
domestic cattle	6000	-20.4	+5.8	3.2
domestic cattle	6000	-20.0	+6.0	3.2
domestic pig	6000	-20.0	+5.7	3.3
field hare	6000	-21.6	+3.8	3.3
brown bear	6000	-22.0	+0.0	3.7
deer	6000	-21.5	+5.9	3.7
PLANTS				
wheat (mono-grain)		-24.6	+4.2	
wheat (two-grain)		-25.3	+2.8	
barley		-25.9	+4.3	
mixture peas		-23.2	+1.9	

Tab. 1. The isotopic composition of carbon and nitrogen of plants and animal remains associated with human skeletons.

figure 4. No significant difference in the dietary habits of the population between the two periods was found. The isotopic composition of carbon in the human samples range between -22.5 and -19.6‰ , while $\delta^{15}\text{N}$ values range from $+4.9$ to $+11.5\text{‰}$. The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values plotted in a single, well-defined cluster suggest a diet that was relatively homogeneous and predominantly based on a purely terrestrial system. In other words, essentially all of the protein in the diet over at least 10 or so years of their lives came from the terrestrial system. The very uniform $\delta^{15}\text{N}$ values, around 9‰ through this period, are indicative of a population obtaining most of its protein from herbivores, domestic and wild animals, and relatively little from plant foods. Similar values are observed in Neolithic human remains from southern Portugal (Straus *et al.* 1992; Lubell *et al.* 1994). Whatever the case, there is no good linear correlation between $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values. A simple explanation of the linear trend of these two values is that the individuals whose collagen is plotted along the line were eating varying proportions from only two isotopically distinct, homogeneous food sources. So, the data suggests that diets were not homogeneous and there was a de-

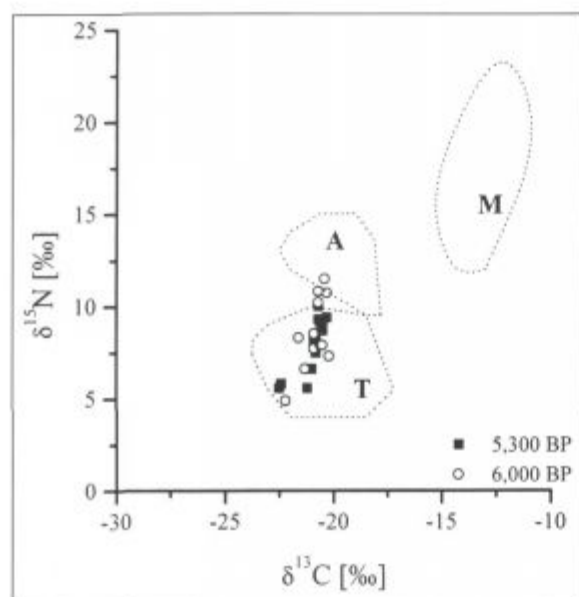


Fig. 4. Scatter diagram of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in human samples from Ajdovska jama. The ranges of aquatic carnivores and omnivores (A), marine carnivores and omnivores (M) and terrestrial foods (T) established by Schwarcz (1991) (+5‰ for $\delta^{13}\text{C}$ and +3‰ for $\delta^{15}\text{N}$ is added to arrive at consumer values).

crease in the diversity of food choices during both periods in the Neolithic. The results indicate different nutritional habits among the Neolithic population in the central part of Europe in comparison with the study of Bonsall et al. (1997). The results of this study, which includes the Mesolithic and also the earliest Neolithic inhabitants, suggest that in the Mesolithic period people had high protein diets derived mostly from riverine food sources. A shift in dietary patterns occurred between 7600 and 7300 BP, reflecting the intake of higher proportions of terrestrial food. It seems that changes coincide with the introduction of cultivation in the Iron Gates, which was not so dramatic as that seen in some other areas of Europe, such as Portugal (Straus et al. 1992; Lubell et al. 1994). Traditional food sources were not abandoned in favour of agricultural produce.

Comparisons of the stable isotopic ratios of collagen from males, females and children are presented graphically in figure 5. It is seen from the results that in a given population with a range of possible foods, the individuals in the population have individual, personal, preferences in diet which in turn determine their individual δ -values. The different nutritional habits of individuals can be seen more clearly from the $\delta^{15}\text{N}$ values ranged from +4.9 to +11.5‰. The lowest values of $\delta^{15}\text{N}$ found in human collagen and average $\delta^{15}\text{N}$ value of $6.1 \pm 0.9\%$ for domestic

Sample	Age (yr BP)	$\delta^{13}\text{C}$ [‰]	$\delta^{15}\text{N}$ [‰]	C/N
HUMAN				
Male	5300	-22.5	+5.6	2.9
Male	5300	-21.2	+5.6	3.4
Male	5300	-20.5	+9.0	3.5
Female	5300	-20.9	+8.1	3.4
Female	5300	-20.7	+9.3	3.4
Female	5300	-20.5	+8.7	3.3
Child (boy 10-12 yrs)	5300	-20.3	+9.4	3.2
Child (boy 10 yrs)	5300	-20.8	+7.5	2.9
Child (7-8 yrs)	5300	-20.8	+8.2	2.8
Child (boy 6 yrs)	5300	-22.4	+5.8	3.5
Child (6 yrs)	5300	-20.6	+9.1	3.0
Child (4 yrs)	5300	-21.0	+6.6	3.2
Child (2 yrs)	5300	-20.7	+10.0	3.3
Male	6000	-22.2	+4.9	3.2
Male	6000	-20.9	+7.7	3.2
Male	6000	-20.6	+7.9	3.3
Female	6000	-20.9	+8.5	3.2
Female	6000	-20.5	+7.9	3.2
Female	6000	-20.2	+7.3	3.3
Child (6-7 yrs)	6000	-21.6	+8.3	3.6
Child (5 yrs)	6000	-20.3	+10.7	3.5
Child (boy 4 yrs)	6000	-21.3	+6.6	3.6
Child (1-2 yrs)	6000	-20.7	+10.2	2.9
Child (1-2 yrs)	6000	-20.4	+11.5	3.5
Child (1 yr)	6000	-20.7	+10.8	3.5

Tab. 2. The collagen stable isotope values in human skeletons. The table includes the amount of carbon vs. nitrogen in the extracted collagen samples are collected.

animal collagen, fit reasonably well with the expected values of 6‰ for vegetarian humans and herbivores, respectively. It is interesting that the lowest values are found in males' collagen, and there is a tendency for females to be associated with higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Fig. 5), indicating that females' diet was based mainly on meat from domestic animals. This difference in the diets of males and females can arise for a variety of reasons related to economic or social customs as well as to biological need or health problems. Men and women often eat different foods because of food taboos, or because members of one sex are guaranteed preferential access to certain foods. During pregnancy and lactation, women's protein needs increase: pregnant women may consume more meat and have higher protein intake, than is the norm for men and non-pregnant women. Moreover, at this time metabolic turnover is higher and food eaten may be exaggerated in the overall collagen signal. Other reasons for the

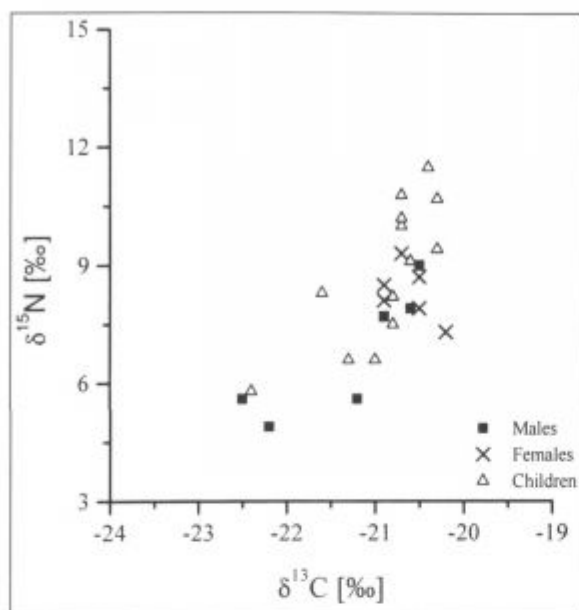


Fig. 5. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of 'Neolithic' males, females and children from Ajdovska jama.

difference in nutritional habits may be related to different food supply activities. It is very difficult to see how the nutritional demands of pregnancy or the division of labour could account for the differences seen in men and women.

Comparing the results of adults and children also indicates significant differences. The highest isotopic composition of nitrogen was found in the bones of one- and two-year-old children. The values are approximately 3‰ higher than those found in the female bones, indicating the "weaning effect". These results could not be compared with other studies, because there is no data available on the bone samples of children at the same age until now. What is more remarkable is that the infants between 4 and 10 years old have lower $\delta^{15}\text{N}$ values in comparison with children one to two years old. These results

indicate a new dietary regime that has been identified in the isotopic composition of collagen in a short period over two and ten years. The most probable reason is that in children bone collagen deposition has a very high turnover rate in comparison to adults. Therefore, bone chemistry changes quickly and can reflect new nutrition habits. A difference between older children is also observed. From the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (approx. -22‰ and +6‰, respectively) it is seen that the protein consumed by some children is based mostly on plants, most probably on cereals, while the diet of other children favours meat from domesticated animals. The results correlate extremely well with the hypothesised model of human diets calculated by R. Schulting (1998, *Tab. 1*).

In conclusion it is worth noting that the stable isotope evidence suggests that the Neolithic population living in Slovenia had individual, personal preferences in diet in which the bulk of the protein was derived from terrestrial food sources. This diet was based mostly on herbivores, domestic and wild animals and relatively little on plant foods. The most interesting and original results are obtained in infants and young children. The significant higher $\delta^{15}\text{N}$ values certainly relate to "weaning effects", while older children had new dietary habits which markedly changed the isotopic signature of bone collagen in a relatively short period.

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