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# Highly bioavailable α-linolenic acid from the subcutaneous fat of the Palaeolithic Relict "Galician horse"

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**Abstract**

The subcutaneous fat of horses stores considerable amounts of α-linolenic acid, which could have contributed to fulfil the daily needs of omega-3 for hominins at the Upper Palaeolithic. To discern the scope of this possibility, several muscles and the subcutaneous fat of Galician horse, a horse relict of the Ice Age, have been studied. The results indicate that grass-fed Galician horse contains in the subcutaneous fat highly bioavailable α-linolenic acid (24% of total fatty acids), thus reducing the needs of other sources to fulfil the daily intake of omega-3, for current and Palaeolithic humans. In addition, the possible use of carcass by Palaeolithic humans hunters/scavengers is clarified through the study of fat degradation under environmental conditions, and toxic compounds generated in the brain indicates that the behaviour of scavengers to consume brains was possible for men of the Ice Ages, but not for any hominin at tropical temperatures.



## Introduction

Nowadays, it is widely recognized that fat is essential for human nourishment (Burr and Burr, 1929), specifically, the fatty acids (FAs), some of them being essentials (EFAs), and thus playing a critical role for the maintenance of several physiological functions in the body. The active EFAs belong to two well differentiated families, *n*-3 (omega-3) and n-6 (omega-6). The *n*-6 FAs include linoleic acid (LA, 18:2*n*-6), and its active derivative, arachidonic acid (AA, 20:4*n*-6), while *n*-3 have the dietary precursor α-linolenic acid (ALA, 18:3*n*-3) and the metabolically biosynthesized eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3). Both *n*-3 and *n*-6 derivatives can be obtained through several metabolic steps from their precursors via desaturation and elongation, and thus the easiest way to ensure the recommend daily allowance is by eating foods containing adequate amounts of both ALA and LA, because the bioconversion of LA and ALA to their higher chain homologues in humans depends on the ratio of ingested *n*-6 and *n*-3 FAs (Guil-Guerrero, 2007; Harnack *et al*., 2009).

For modern humans, the presence of some EFAs in the diet is critical, as it has been reported that Western diet lacks of adequate amounts of *n*-3 FAs, showing a *n*-6/*n*-3 ratio of 15-20/1, instead of 1/1, which was present in the diet of our more recent human ancestors (Eaton and Konner, 1985; Connor *et al*., 1992). Recommended intakes range from 250 mg/day to 610 mg/day of EPA+DHA for adults (Table 1). ALA, the physiological precursor of *n*-3, is present in some vegetable oils, such as soybean, canola and some nuts, but both the biosynthetic capacity and daily amounts are usually insufficient to achieve an adequate amount of EPA+DHA to meet the recommended daily allowance (Guil-Guerrero, 2007). Moreover, DHA is needed for the development of learning abilities (Horrocks and Yeo, 1999), and for the maintenance of the brain during aging (Lukiw and Bazan, 2008). In addition, there are a large number of pathologies that can be healed or alleviated by ingesting DHA. For example, DHA may reduce the development of unipolar depression (Marangell *et al*., 2003), secondary depression in alcoholism (Mischoulon and Fava, 2000), and multiple sclerosis (Hutter, 1993).

In mammals, there is a close similarity in content of polyunsaturated FAs (PUFAs) in the brain among all species, with DHA and AA being most prominent (Crawford and Sinclair, 1976), and limitations to the supply of either one of these FAs will determine limitations to brain growth (Crawford *et al*., 1999). In this sense, it has been indicated that Upper Palaeolithic humans consumed a diet rich in *n*-3 FAs and low in saturated FAs (SFAs), and a ratio *n*-6 to *n*-3 (*n*-6/*n*-3) of approximately 1, which is very different from the current Western diets, enriched in *n*-6 PUFAs (Simopoulos, 2011).

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Among hominins, in contrast to *Homo* spp., *Australopithecus* spp. shows a low encephalization rate, thus, the process of brain evolution should be focused on the investigation of FA availability for *Homo* spp. For this last hominin, it has been argued that freshwater fish and shellfish would have represented the animal foods that provided the FAs needed for encephalization (Broadhurst *et al*., 1998).

For both *H. erectus* and *H. sapiens* there is now evidence of seafood consumption, so it is supposed that they easily acquired the PUFA needed from fish in order to develop a rapid cerebral expansion (Cordain *et al*., 2000); although this is subject to controversy (Cordain *et al*., 2001; Snodgrass and Leonard, 2009), because fish provides a very low amount of energy.

Neanderthals (*H. neandarthalensis*) evolved in Europe during the Middle Pleistocene (Snodgrass and Leonard, 2009). In this sense, the early Upper Palaeolithic (UP) in Western Europe represented a period of significant niche contraction for humans, probably due to a reduction in the diversity of mammalian species (Morin, 2007). During this period, populations depended mostly on terrestrial mammals for subsistence, whereas marine mammals, lacustrine resources and plant foods seem to have played a marginal role in the diet of these humans (Stiner *et al.*, 1999; Bocherens *et al.*, 2005). This feeding behavior may provide certain advantages to the hunting tribes, taking into account that multiple studies have shown that animal foods almost always result in a higher ratio of energy capture to expenditure than plant/fish-based foods (Hawkes and O'Connell, 1985; Simms, 1987). Nevertheless, this diet may have also provided disadvantages, as indicated by Rudman *et al.* (1973), who showed that the mean maximal rate of urea synthesis (MRUS) in normal subjects is 65 mg N $h^{-1}$•kg body wt -0.75 and that protein intakes that exceeded the MRUS resulted in hyperammonemia and hyperaminoacidemia. Consequently, the preferred solution by most worldwide hunter-gatherers to circumvent the excess of dietary protein would likely have been a relative increase in total dietary fat from animal foods (Pryor, 2008). In this context, for hunter-gatherer societies, probably all of the edible carcasses were processed to obtain fat (Speth and Spielmann, 1983).

At a critical stage as was the UP period, in a situation of recurrent glaciations, when consumption of plants and marine resources were marginal (Morin, 2007), the nutrition of Homo in Europe heavily depended on mammals, among which the horse must have had a crucial role (Levine, 1998). This species was a valuable resource, and its flesh and milk still are very valuable food for human beings, particularly in grassland habitats (Pryor, 2008).

Figure 1. The current Galician horse descends from the *Equus gracilis*, who lived in Central Europe at the Ice Age, and gave rise to the Celtic pony, which in the mountains of the North of Spain evolved into the present day Galician, Asturcón, and Vasco-navarro ponies, which were employed in transport, in war and in religious sacrifices in the antiquity.

Figure 2. Every year during the summer, some days, the villagers go up the mountains in search of the horses, which lead to a stone enclosure where animals are captured to mark them and cut their manes.

The fact that the horse is not a ruminant animal allows the storage of most of the consumed *n*-3 FAs in the subcutaneous fat (SF). This fact was established several years ago by Shorland *et al.* (1952), who noticed that ALA, the main component of pasture FAs, comprised about 17% of the depot fat of New Zealand horses. Although the *n*-3 FA amounts cited in the muscle and fat of horses until now is unusual among animal fats, probably it does not reflect the amount that might have been present in horses of the late Pleistocene, taking into account both biological diversity and feeding differences. Among current horses, the Galician Horse (GH) seems to be the best candidate to establish the capacity for n-3 FAs accumulation of the horses that nourished our human ancestors in the UP period. This horse is descended from *Equus gracilis*, whose habitat was established in Central Europe in the Palaeolithic period, and was the ancestor of the Celtic Pony, which in the Galician mountains remains as GH (Vega *et al*., 1997).

In this context, there still remain important issues to solve in relation to meat consumption and the availability of *n*-3 PUFAs to UP societies: which was the composition of the fat they ingested in relation to *n*-3 PUFAs? Was their diet suitable to meet their

*n*-3 metabolic needs? Would have been possible for Palaeolithic hominins a behaviour of scavengers, bearing in mind that at appropriate environmental temperature a highly unsaturated fat could become toxic very quickly?

This paper tries to provide answers to these questions through the study of nutrient profiles of fat and muscles from the Palaeolithic-relict Galician horse (Figures 1, 2), an animal similar to those that were eaten by our UP ancestors, as well as by analyzing the toxicity of the organs with critical content in PUFAs.

## Materials and Methods

*Samples collection*

Muscles and SF of GHs were obtained from the Experimental Research Centre Parque Tecnolóxico de Galicia, Avenida Galicia nº 4 San Cibrao das Viñas, Ourense España, 32900. Samples of grass-fed GH (Freedom Extensive System, FES), according to an extensive production system on wood pasture in the Galician mountains were obtained from 5 females and 3 males, slaughtered at 2 years old, in good health condition. Gorse (*Ulex europaeus*), broom (*Cytisus scoparius*) and coarse grass were the main component of their diets (Rigueiro Rodríguez *et al*., 2005). Samples of Semi Extensive System (SES) were obtained from horses (4 females and 3 males), slaughtered at 2 years old, in good health status, fed in a pasture until 3 months prior to slaughter. For the last 3 months the horses were finished indoors, on a concentrate ration consisting of hay silage "*ad libitum*" and 3 kg of concentrate (Eco-Feed®) per head per day. Samples were taken from frozen animals, and kept frozen (-18ºC) until analysis.

*Brain fat degradation*

Horse skulls were obtained in the slaughterhouse El Cabezo de la Plata (Murcia, Spain). Three different environments were selected to study the degradation of FAs in the brain of horses. Tropical temperatures experiments (35°C mean) were effected in June outside the laboratory and under sun exposure; room temperature (20°C mean) was obtained inside the laboratory, while environmental conditions for winter experiments (4°C) were obtained inside a refrigerator. For each one of these locations, a sufficient number of flayed skulls were prepared, being sampled daily. For analysis, the skulls were opened, and immediately the brains were homogenised. Sufficient amount of brain were taken to accomplish analyses.

The extraction of the lipids of the brain and the calculation of the K270 extinction coefficient was carried out following the analytical methods previously described (Danopoulos and Ninni,1972) for fish. The K270 extinction coefficient was expressed as the specific extinctions of a 1% (w/v) solution of lipid extract in cyclohexane in 1 cm cell path length, as described in the Regulations EEC/2568/91 of the European Union Commission, and Danopoulos and Ninni (1972).

*Moisture determination*

Moisture was determined by drying a representative 2-g sample in an oven with air circulation at 105ºC for 40 h.

*Lipid determination*

20 g of small cut pieces of SF were homogenized in a Waring blender, with 100 ml of methanol. Then, 50 ml chloroform and 40 ml water were added, and the resulting mixture was homogenized again for 2 min. After that, 50 ml chloroform and 50 ml water were added, and homogenization was newly applied for 30 s. Then, 100 ml of the mixture was transferred to glass tubes and centrifuged (3300 × g, 10°C). The liquids were decanted and collected by filtration through a sintered glass funnel, and kept separate. The resulting solids were extracted again, by adding 20 ml 1:1 (v/v) chloroform:methanol, and both resulting extracts were combined and transferred into a separatory funnel. The chloroform layer was passed through a 2.5-cm thick layer of anhydrous sodium sulphate using Whatman No. 1 filter paper in a funnel. Finally, the solvents were removed by using a rotary evaporator under vacuum, at 40°C, and the content of lipids in the samples were calculated by weight difference (Folch *et al*., 1957).

*Fatty acids determination*

50 mg of each dried sample were weighted in test tubes and *n*-hexane (1 mL) was added to each. FA methyl esters (FAMEs) were obtained after adding 1 mL of the methylation mixture, which was composed by heating methanol:acetyl chloride (20:1 v/v), at 100 ºC for 10 min. After cooling at room temperature, 1 mL of distilled water was added to each tube, and after that the tubes were centrifuged at 3500 rpm for 5 min. The upper hexane layer was removed for Gas-Liquid Chromatography (GLC) analysis (Lepage and Roy, 1984).

*GLC analyses*

FAMEs were analyzed by using a Focus GLC (Thermo Electron, Cambridge, UK) equipped with flame ionization detector and a Omegawax 250 capillary column (30 m x 0,25 mm i. d. x 0,25 μm film thickness; Supelco, Bellefonte, PA, USA). The temperature programme was: 1 min at 90ºC,

heating until 200ºC at a rate of 10ºC/min, constant temperature at 200ºC (3 min), heating until 260ºC at a rate of 6ºC/min and constant temperature at 260ºC (5 min). Injector temperature was 250ºC with split ratio 50:1. Injection volume was 4 µL. Detector temperature was 260ºC. Nitrogen was used as carrier gas (1 mL/min). Peaks were identified by retention times obtained for known FAME standards (PUFAs No. 1 from Sigma, St. Louis, USA) and FA contents were estimated by using methyl pentadecanoate (15:0) as internal standard.

Peaks were identified by retention times obtained for known FAME standards (PUFAs No. 1, 47033; methyl γ-linolenate 98.5% purity, L6503; and methyl stearidonate 97% purity, 43959 FLUKA) from Sigma, (St. Louis, USA), while FA contents were estimated by using methyl pentadecanoate (15:0; 99.5% purity; 76560 Fluka) from Sigma as internal standard.

All samples were subjected to a second round of analyses by GLC-mass spectrometry (GLC-MS) at the "Scientific Instrumentation Centre" of the Universidad de Granada. Samples were injected (2 µl) in an Agilent 7890A gas chromatographer equipped with an apolar column in split mode, coupled with a Quattro micro GC mass spectrophotometer (Waters, UK), with a positive electron impact source (70 eV) and full scan spectra acquisition.

*Hydrolysis of the lipid fraction of the subcutaneous fat*

SF was extracted with *n*-hexane to obtain a suitable fraction for enzymatic essays. This fraction was enzymatically hydrolysed as previously described (López López *et al*., 2001). Briefly, 50 mg of extracted fat was hydrolyzed by adding porcine pancreatic lipase (40 mg) in 4 ml of a Tris solution (1M, pH = 8.0), 1 ml of a sodium cholate solution (0.1% w/v) and 400 µl of a calcium chloride solution (22% w/v). The flask was then placed in a water bath (37ºC, 120 rpm) for 40 min. After that, it was removed and cooled in a water bath at 20ºC and 2 ml of hydrochloric acid (6 M) and 1 ml of *n*-hexane were added. The flask was shaken for 1 min and the content was transferred to test tubes for centrifugation (3500 rpm, 5 min). The clear hexane top layer containing the hydrolysis products was transferred into a 10 ml-test tube. Hydrolysis reaction was performed in duplicate. An aliquot was collected to separate the hydrolysis products by TLC as previously described (Rincón-Cervera and Guil-Guerrero, 2010). All bands were scrapped off and methylated with 1 mg internal standard pentadecanoic acid, 15:0). Amounts of each lipid class were quantified by GLC. Methylation process and GLC analysis are described above.

*Ash*

It was determined by incineration of a representative 0.5 g sample in an oven at 450ºC for 48 h (Rebolloso-Fuentes *et al*., 2000).

*Crude protein*

Total nitrogen was determined by means of an elemental analyzer (Leco CHNS-932). The carrier gas was He, while oxygen was the burning gas (Rebolloso-Fuentes *et al*., 2000).

*Energy*

It was computed by multiplying the values obtained for protein and lipids by 4.00 and 9.00 respectively, and adding up the values (Rebolloso-Fuentes *et al*., 2000).

*Statistical analysis*

Experimental results were mean ± S.D. A Multifactorial Analysis of the Variance (ANOVA, Statgraphics Plus 5.1 for Windows, Manugistics, Inc., Rockville, MA, USA) was effected trough the data obtained from each sample. P values $< 0.05$ were regarded as significant. Experiments for all samples were conducted in triplicate.

## Results and Discussion

*Fatty acid profiles of galician horse*

The results of the analyses of FAs carried out in SES and FES are shown in Table 2. Notice that the SF shows an unusual amount of ALA (24.3% of total FAs) for FES, in contrast with the low percentage found for the samples taken from SES (7.6%). From data in Table 2, it is also clear that by consuming meat, the low percentage of *n*-3 PUFAs contained therein means that this organ is unable to meet the daily requirements. Conversely, the SF shows a high content of ALA, due to the accumulation of the FAs from the natural pasture, in which ALA represents the main FA (Shorland *et al*., 1952), although in comparison with other horses eating grass (Mordovskaya *et al*., 2006), the SF of this horse reaches higher percentages of ALA, so there are probably genetic factors to consider. In addition, the ancestor of this horse could have had a more complex and advantageous FA profile, taking into account that he lived in the Ice Age, and consumed a different pasture to that is observed today for GH, which was probably similar to that is noted for the actual reindeer and musk deer, that feed on several lichens such as *Claudine* spp. and *Cretaria* spp., species that contains both EPA and DHA, as well as other *n*-3 PUFAs in tissues (Sampels, 2005).

Table 1. Recommended daily intakes ff EPA+DHA made by different organizations

| Organization | Recommended Daily Intakes |
|---|---|
| The International Society for the Study of Fatty Acids and Lipids (ISSFAL) | 500 mg EPA/DHA |
| The National Institute of Health (NIH) | 650 mg EPA/DHA (minimum 220 mg of each) |
| The National Health and Medicinal Research Council | 610 mg EPA/DHA/DPA for men and 430 mg for women |
| European Food Safety Authority (EFSA) | 250 mg EPA/DHA |

The FA profile of a single muscle of this horse has been recently reported (Lorenzo *et al*., 2010). Authors report the partial differences in intramuscular FAs of *Longissimus dorsi* in GH foals, considering FES vs. SES. In that work, it is indicates that the feeding system had significant differences on PUFA content, which was higher in FES than in SES, and also that the major PUFA content in FES muscle was ALA, which reached a 23.87% of total FAs. Unfortunately, authors did not provide the total amount of FA in muscle, nor the FA profile of different muscles from foals, nor the FA profile of the SF. Therefore, until the present work, the scope of the benefits of the GHs as a source of *n*-3 PUFAs still remained unknown.

*Bioavailability of α-linolenic acid from the subcutaneous fat of Galician horse*

The bioavailability of ALA in SF needs to be confirmed, because the bioavailability of EFAs from some fats is sometimes low. This way, when triglycerides (TGs) are processed by the human metabolism, the FAs present in position *sn*-1 and *sn*-3 of the glycerol backbone are released by pancreatic lipase whereas the FAs esterified in position *sn*-2 remain unreleased, and are absorbed by intestinal mucosa as *sn*-2 monoglycerides. So, PUFAs that are esterified in *sn*-2 position of TGs are more easily adsorbed than those which are in *sn*-1 and *sn*-3 positions, and therefore are more bioavailable for metabolic needs (Iwasaki and Yamane, 2000). The regioespecific analyses of the TGs of the SF of GH from SES and FES is exposed in Table 3. Notice that ALA is present much more often in the *sn*-2 position of the glycerol than in the *sn*-1,3 position, improving its percentage from 23.9% of total FAs to 34.5% of total FAs in the *sn*-2 position. This result is a good indicator of ALA bioavailability, when compared with other ALA rich lipids. For example, soybean oil (9.3% ALA on total FAs) has in the *sn*-2 position 10.8% ALA of total FAs (Bracco, 1994). For SES horses the results were less than for FES, and therefore the hypothesis about the nutritional benefits of the SF of grass-fed horses is corroborated.

*The daily needs of n-3 PUFAs can be fulfilled with the subcutaneous fat of horses, not with Muscles*

Considering both the ALA percentage of total FAs in the SF of SES horses (24.3%), and the total FAs content (60.5%), it is expected an average of ALA content in this organ of 14.7%. From this figure, it is difficult to know the exact amount of both EPA and DHA that can be biosynthesized: ALA has the highest rate of oxidation among all PUFA, thus only a small proportion (1%) is metabolized to DHA (Goyens *et al*., 2005). However, considerable variability in the conversion rates among individuals have been reported, and this figure varies widely depending on several factors, such as intake of *n*-6 PUFA, age, gender, and so on (Arterburn *et al*., 2006; Harnack *et al.*, 2009). In any case, considering the established average value of 1%, it is expected to obtain 147 mg of EPA+DHA by consuming 100 g of the SF of GH. Thus, to get the minimal recommended daily allowance of 250 mg of EPA+DHA (Table 1), 170 g of SF seem to be enough, although variable amounts of the targeted FAs can be ingested by consuming lean meat. Probably, for Palaeolithic men, the amount of SF needed to meet the daily allowance for both FAs could be lower than other shown here, by considering the above exposed reasons about feeding of the primitive Palaeolithic horses; in this sense, a comparison of ALA percentages found in the SF between SES and FES animals (Table 2) corroborates this idea.

Despite the relative good concentration of EPA+DHA that could be formed from SF intake, the above made calculation is very conservative. Currently, although there is wide evidence that the intake of ALA will lead to increased amounts of EPA and DHA, it is necessary to take into account the amount of LA in the diet, as the conversion of ALA to EPA+DHA decreases with high dietary ratios of LA:ALA (Arterburn *et al.*, 2006; Harnack *et al.*, 2009; Brenna *et al*., 2009). Thus, given the low amount of LA that the SF of FES horses contains (Table 2), the bioconversion of ALA to EPA+DHA might be higher than expected.

By considering the needs of EPA+DHA for of an entire social group from 10 to 30 individuals, as hunters are organized today, and the amount of 7 kg of SF average for a typical horse (Martin-Rosset *et al*., 2008), by hunting a single horse, the social group could fulfil the daily needs of *n*-3 PUFAs for a 1-4 days period, which is compatible with the hunting behaviour of the modern hunter-gatherer societies.

Looking at values for ALA in several muscles shown in Table 2, is apparent to consider that the daily needs of EPA+DHA are unattainable by consuming only horse muscles. Although the muscles of horses from FES had relative high percentages of ALA, higher than those values found in muscles from

Table 2. Fatty acid composition (fatty acid % of total fatty acids)of several muscles and subcutaneous fat of Galician horses*

| | Livestock production system (n=7) | | | | Freedom extensive system (n=8) | | | |
|---|---|---|---|---|---|---|---|---|
| FA | *Longissimus dorsii* | Deltoides | *Serratus ventralis* | SF (abdominal) | *Longissimus dorsii* | Deltoides | *Serratus ventralis* | SF (abdominal) |
| 12:0 | - | 0.8 ± 0.1 [a] | - | 1.8 ± 0.1 [b] | - | 0.5 ± 0.1 [c] | - | 0.2 ± 0.1 [d] |
| 14:0 | 0.15 ± 0.2 [a] | 1.8 ± 0.2b [a] | 0.5 ± 0.2 [c] | 6.8 ± 0.2 [d] | 0.35 ± 0.2 [c] | 1.2 ± 0.3 [d] | 0.3 ± 0 .1 [c] | 3.1 ± 0.1 [e] |
| 16:0 | 17.6 ± 0.4 [a] | 17.9 ± 0.3 [a] | 20.3 ± 0.6 [b] | 27.9 ± 0.6 [c] | 15.8 ± 0.6 [d] | 13.4 ± 0.4 [e] | 15.9 ± 0.4 [d] | 23.4 ± 0.3 [f] |
| 16:1*n*-7 | 0.7 ± 0.1 [a] | 3.1 ± 0.1 [b] | 1.6 ± 0.2 [c] | 5.5 ± 0.2 [d] | 0.8 ± 0.1 [a] | 2.9 ± 0.1 [b] | 0.9 ± 0.1 [a] | 2.8 ± 0.1 [b] |
| 18:0 | 10.0 ± 0.6 [a] | 9.4 ± 0.4 [a] | 9.9 ± 0.4 [a] | 4.6 ± 0.1 [b] | 10.7 ± 0.1 [a] | 7.7 ± 0.1 [c] | 10.8 ± 0.1 [a] | 7.4 ± 0.1 [c] |
| 18:1*n*-9 | 6.3 ± 0.3 [a] | 14.6 ± 0.2 [b] | 10.2 ± 0.3 [c] | 25.1 ± 0.4 [d] | 7.7 ± 0.4 [d] | 14.3 ± 0.4 [b] | 8.7 ± 0.8 [d] | 27.5 ± 0.4 [d] |
| 18:1*n*-7 | 2.0 ± 0.1 [a] | 2.2 ± 0.1 [a] | 2.2 ± 0.1 [a] | 0.7 ± 0.1 [b] | 1.9 ± 0.1 [a] | 3.0 ± 0.1 [c] | 1.8 ± 0.1 [a] | 0.6 ± 0.4 [b] |
| 18:2*n*-6 | 32.4 ± 1.3 [a] | 27.1 ± 0.1 [b] | 32.8 ± 0.4 [a] | 15.9 ± 0.1 [c] | 29.9 ± 0.4 [d] | 23.3 ± 0.1 [c] | 30.9 ± 0.8 [d] | 7.6 ± 0.0 [e] |
| 18:3*n*-3 | 0.9 ± 0.0 | 7.2 ± 0.1 | 1.9 ± 0.2 | 7.6 ± 0.1 | 4.5 ± 0.1 | 12.3 ± 0.4 | 6.6 ± 0.2 | 24.3 ± 0.1 |
| 20:1*n*-9 | - | - | 0.2 ± 0.1 [a] | 0.4 ± 0.1 [b] | - | - | - | 0.4 ± 0.1 [b] |
| 20:4*n*-6 | 6.7 ± 0.4 [a] | 4.0 ± 0.3 [b] | 5.0 ± 0.1 [c] | - | 5.8 ± 0.1 [d] | 4.6 ± 0.2 [e] | 4.6 ± 0.2 [e] | - |
| 20:5*n*-3 | 0.9 ± 0.1 [a] | - | 0.8 ± 0.1 [a] | - | 1.7 ± 0.1 [b] | 0.9 ± 0.1 [a] | 1.4 ± 0.1 [c] | - |
| 22:5*n*-3 | - | 1.6 ± 0.1 [a] | 2.4 ± 0.1 [b] | 0.2 ± 0.1 [c] | 3.3 ± 0.1 [d] | 2.6 ± 0.2 [b] | 2.7 ± 0.1 [b] | 0.2 ± 0.1 [c] |
| 22:6*n*-3 | 0.5 ± 0.3 [a] | - | 0.7 ± 0.2 [a] | - | 0.8 ± 0.1 [a] | 0.7 ± 0.1 [a] | 0.8 ± 0.1 [a] | - |
| Others | 19.4 ± 0.7 [a] | 10.5 ± 0.2 [b] | 11.85 ± 0.4 [c] | 3.5 ± 0.1 [d] | 16.9 ± 1.0 [e] | 13.1 ± 1.1 [c] | 15.0 ± 0.4 [f] | 2.6 ± 0.3 [g] |
| TotalFAs** | 0.6 ± 0.1 [a] | 0.5 ± 0.1 [a] | 0.5 ± 0.1 [a] | 63.7 ± 3.1 [b] | 0.7 ± 0.1 [a] | 0.7 ± 0.1 [a] | 0.8 ± 0.1 [a] | 60.5 ± 2.0 [b] |

*Values within a file followed by the same superscript letter were not significantly different ($p \leq 0.05$) by the Duncan's Multiple Range Test.
**g FAs/100 g fresh tissue

Table 3. Regiospecific analysis for the more prominent fatty acids of the subcutaneous fat of Galician horses (fatty acids % of total fatty acids)

| | Total Fatty Acids | | *sn*-2 | | *sn*-1,3 | |
|---|---|---|---|---|---|---|
| | Freedom (n=8) | Livestock (n=7) | Freedom (n=8) | Livestock (n=7) | Freedom (n=8) | Livestock (n=7) |
| 16:0 | 23.9 ± 0.6 [a] | 26.3 ± 0.1 [b] | 6.9 ± 0.7 [c] | 7.9 ± 0.4 [c] | 32.4 ± 0.5 [d] | 35.6 ± 0.1 [e] |
| 18:1*n*-9 | 28.1 ± 0.1 [a] | 28.3 ± 0.2 [a] | 31.6 ± 0.6 [b] | 29.6 ± 0.5 [c] | 26.3 ± 0.2 [d] | 27.6 ± 0.6 [e] |
| 18:2*n*-6 | 7.1 ± 0.1 [a] | 16.6 ± 0.1 [b] | 14.9 ± 0.2 [a] | 33.7 ± 0.1 [c] | 3.2 ± 0.1 [d] | 8.1 ± 0.2 [e] |
| 18:3*n*-3 | 23.9 ± 011 [a] | 7.1 ± 0.1 [b] | 34.5 ± 1.7 [c] | 10.1 ± 0.1 [d] | 18.7 ± 0.1 [e] | 5.5 ± 0.2 [f] |

*Values within a file followed by the same superscript letter were not significantly different ($p \leq 0.05$) by the Duncan's Multiple Range Test.

SES, by considering the extreme low percentage of total FAs shown in by all muscles for animals from both production system types (Table 2), EPA+DHA derived from ALA reaches negligible amounts. Also, the small figures for EPA and DHA shown in all tested muscles allow the unique possibility of meeting the daily allowance of these FAs by consuming the SF.

In this context, several authors provide information about the very small amounts of DHA or EPA in the muscle of grass fed animals usually eaten by UP men: for red deer it has been cited ≈0.3-0.8% DHA of total FAs (Polak *et al*., 2008); for reindeer, Finstad *et al.* (2007) have found small amounts of DHA in muscle (0.1-0.3% of total FAs); for range bison, DHA has been detected reaching extremely low concentrations (≈0.2% of total FAs) with a total FAs of ≈10-20 mg/100 g in several edible muscles (Rule *et al*., 2002); for the common cow, descended from the auroch (*Bos taurus primigenius*), a species usually eaten by Palaeolithic men (Snodgrass and Leonard, 2009), in an extensive review, Daley *et al.* (2010) found low percentages of DHA: from trace to a 0.4% of total fat, while for EPA the figures are very variable, but are usually less than 1% of total FAs.

Thus, the intake of SF obtained from simple-stomached animals, simultaneously or not with the meat, must have been a common practice for Palaeolithic hominins, which would help to complete the daily requirement of *n*-3 FAs to obtain clean energy, nitrogen-free. This is so because when animal foods are plentiful, and especially when plant foods are concurrently scarce, hunters tend to emphasize the use of high-fat adipose tissue, organ meats, brain, and marrow (Eaton *et al*., 1998). Therefore, muscle tissues would have been a relatively good source of AA (Table 2), while the SF from monogastric animals, such as horses, constituted a raw source of *n*-3 PUFA for Palaeolithic hominins.

Regarding the data about proximate composition of the SF (Table 3), it is possible to calculate the energy derived from the daily intake of 170 g of SF, which is 1073 kcal. This contribution of energy from

Table 4. Proximate composition of the subcutaneous fat of Galician horses (g/100 g fresh tissue)

| System lifestyle | Moisture | Crude Protein | Lipids | Saponifiable oil | Ash | Energy (kcal) |
|---|---|---|---|---|---|---|
| Freedom Extensive System (n=8) | 22.6 ± 1.6 [a] | 12.5 ± 1.1 [a] | 64.6 ± 4.1 [a] | 60.5 ± 1.9 [a] | 0.3 ± 0.1 [a] | 631.4 [a] |
| Livestock Production System (n=7) | 17.3 ± 3.0 [b] | 13.6 ± 1.0 [a] | 69.1 ± 4.7 [a] | 63.7 ± 2.1 [a] | 0.3 ± 0.1 [a] | 676.3 [a] |

*Values within a column followed by the same superscript letter were not significantly different ($p < 0.05$) by the Duncan's Multiple Range Test.

SF is interesting, because it reduces the daily intake of meat needed for Palaeolithic men. In this sense, by comparing energy requirement between hunting in Ice Ages with other similar groups such as Canadian Eskimos hunting caribou, energy expenditure would be approximately 3600 kcal. To meet this energy need, a hunter would have to consume between 3.4 and 3.6 kg of lean meat per day, amounts that for the reasons outlined above would be toxic to hominins (Rudman *et al*., 1973; Speth, 1989), thus, whether hominids hunted or scavenged large mammals their principal target would have been lipids, not protein (Speth, 1989). Therefore, the inclusion of SF from horses in the diet allows accomplishing two important functions: providing "clean energy" while providing the *n*-3 PUFAs needed.

*The use of brain as source of n-3 fatty acids*

Probably, Palaeolithic men did not only consume SF and lean meat. Anthropological and ethnographic data indicate that Stone Age humans consumed not just muscle tissue, but relished certain fatty portions of the carcass including brain and marrow (Stiner, 1991; Silberbauer), being proposed the scavenged brain tissue of ruminants as a rich source of both DHA and AA for encephalization (Cordain *et al.*, 2001, 2002). Regarding the high amount of DHA found in all ruminant brains, this organ might provide a valuable contribution to accomplish the daily allowance of EPA+DHA. But considering the small size that it reaches in all species consumed by primitive men, it seem to be an erratic contribution to EPA+DHA intake, and thus is very difficult to quantify. Any case, the contribution of brain to daily EPA+DHA needs seems to be marginal. An analysis of the homogenate of a full brain of horse (200 g mean) shows an amount of 8% FAs, with 8% DHA, which represents 1.28 g DHA in the brain for a single horse. This figure is enough to fulfill the daily allowance of 3-4 individuals, but not for an entire social group from 10 to 30 people, as exposed above.

*The fat of brains could have been toxic for scavenger Hominins*

The contribution of carcasses as source of SF to the daily intake of EPA+DHA would have been important if a behavior of scavengers for Paleolithic men is considered. Paleolithic hunters, in addition to return to their camps with their killed prey, could

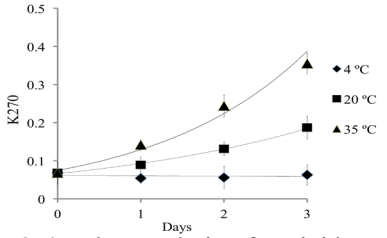


Figure 3. K270 Index Evolution for Lipids Extracted from Horse Brains at Different Temperatures

have returned with animal carcasses partially eaten by other predators, which include SF and skulls (Stiner, 1991; Grayson and Delpech, 1994). Such behavior is consistent with the high intake of SF needed to meet the daily intake of EPA+DHA. However, the toxicity of the fat contained in the SF of the carcasses, especially in skulls, might be an important problem to consider. Logically, there is a variable period between the animal's deaths until consuming by humans, in that the fat could produce toxic compounds, thus preventing the development of human behavior as occasional scavengers.

To discern possible toxicities, the more susceptible organ to oxidation of the carcasses, the brain, which has a high PUFA content, was analyzed in order to determine whether its use was possible by human scavengers of the Palaeolithic. For this purpose, the UV absorbance test at 270 nm has been chosen. This test is generally performed to identify the age of fats, being a precise indicator of oxidation. Absorbance at 270 nm is caused by carbonyl compounds (an intermediate stage of the oxidation) and conjugate trienes (final stages of the oxidation), that are clearly toxic compounds (Wiesman, 2009). The results of the analyses of the K270 index of the brain of several skulls of horses stored at different temperatures and sampled daily are shown in Figure 3. Notice that this index increases quickly from the first day of storage for skulls stored at temperatures higher than 4ºC, reaching values close to 0.4 after three days at 35ºC storage. This last value is much higher than 0.25, the specified limit for edible oils (Biffoli, 1990), thus this fat becomes potentially toxic for humans. Experimental results are in good agreement with previous reports on the stability and distribution of PUFA in rat tissues using the same parameters (Abdelsalam, 2008). In any case, the sensorial characteristics of the skull kept at temperatures above 4ºC after one day of storage lead us to think that it would be very difficult to consume

for any hominin.

Thus, considering the average temperatures for the Ice Ages equal or lower than 4ºC, it seems that scavenger hominins were able to eat all of the organs of carcasses. But looking at data here, and considering other evolutionary times prior to those investigated in this article, the consuming of skulls was probably only occasional for early hominids in Africa. Considering the high temperatures of the African savannah, and considering the long waiting time to get the skulls of herbivores killed by predators due to the rigid and hierarchical protocol of pray consumption by different opportunistic carnivores, the fat of the brain probably would be highly oxidized when obtained by hominids. Moreover, the small size of the brain would make it very difficult to achieve an equivalent consumption among members of any human group.

This means that the collection of skulls would be occasional. Nevertheless, Cordain *et al.* (2001) states that the consumption of brain would have been the most reliable and concentrated source of DHA and AA to early hominids, assuming a behavior of scavengers, and discarding fish, taking into account their low energy density, and also discarding the SF, by considering that it does not contain EPA or DHA. However, although the consumption of the brain was probably practiced by early human scavengers, in addition to this organ is likely that our ancestors in Africa also used the SF of carcasses of zebra (*Equus burchelli*) as *n*-3 source. In this sense, it has been cited that ALA reached 47.5% of total FAs in the adipose tissue of zebra (Williams *et al*., 1987). Thus, this organ may be the energy-dense food that provides the daily needs of EPA+DHA to primitive human scavengers in Africa. Although the SF is often partially consumed by carnivores, the fact that zebra represents the more frequent carcass in the African savannah (Schaller and Lowther, 1969), leads to this equine to be the best candidate that provide both energy dense and *n*-3 FAs to early hominins.

Finally, is timely to consider that other sources of *n*-3 FAs might have been available for Palaeolithic men in addition to the above cited. For example, during the UP in Europe there were populations of Neanderthals who lived on the coast who partially ate marine resources (Richards *et al.*, 2005), thus probably obtaining some *n*-3 of marine origin. However, as indicated by Carlson and Kingston (2007), the regular consumption of aquatic resources rich in preformed DHA may not have been essential. Furthermore, hunter-scavengers of the Ice Age could have some sources of *n*-3 in addition to those shown here and not yet discussed, such as the SF of mammoths and woolly rhinoceros, two monogastric animals that probably have similar *n*-3 enriched FA profile, taking into account both similarities in their digestive apparatus with respect to those of horses, and feeding behavior.

## Conclusions

In this study, the knowledge about *n*-3 availability for humans who lived in the UP Period has been improved. By a series of experiments, we found appropriate answers to different questions related to human nutrition in this critical period. By analyzing a horse similar to another that lived in the Ice Ages, it is demonstrated that its subcutaneous fat is a good source of ALA, and that it could contribute to the recommended daily allowance for EPA and DHA. Also, we found that this ALA source is highly bioavailable. The analysis of the toxicity of the brain due to fat degradation over time showed that the behaviour of scavengers to get the SF and skulls was possible for men in the Ice Ages, but that the brains was probably toxic for early Palaeolithic men in Africa, and zebra carcasses might have been the more prominent source of *n*-3 FAs for African Hominids.

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