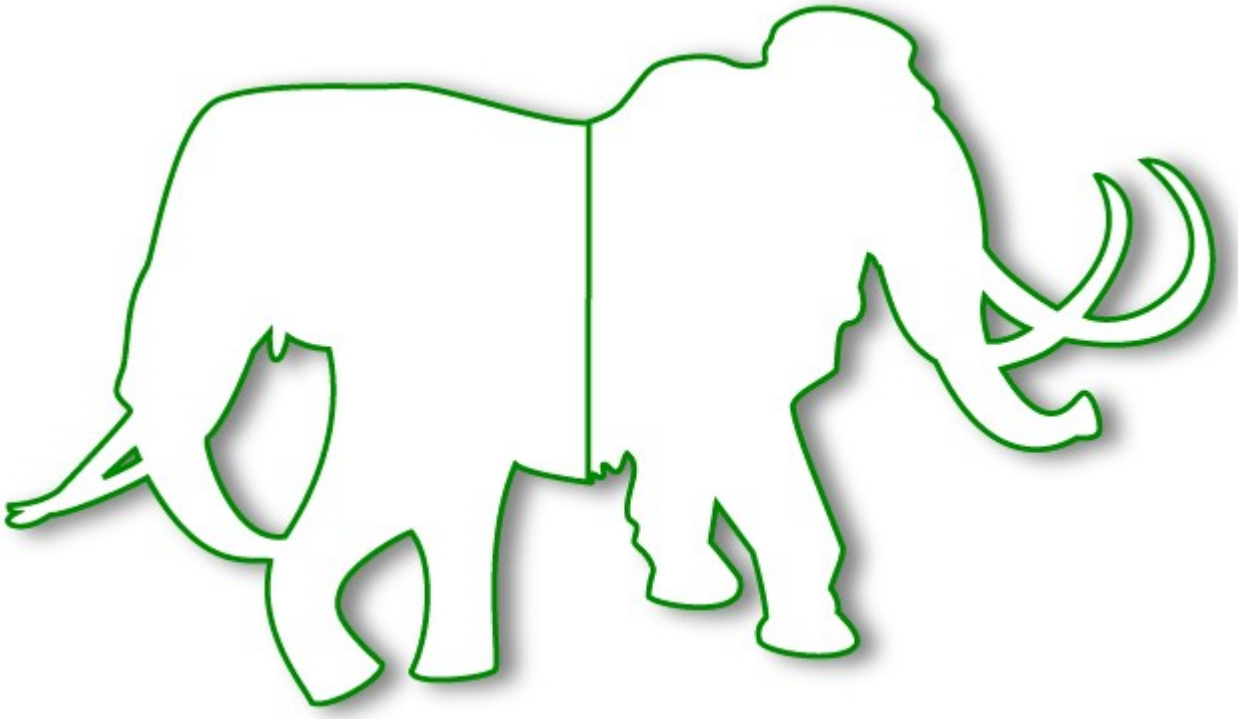
Master thesis Archaeology, specialisation *Science Based Archaeology*, Leiden University

Comparison of Stable Isotope Ratios in Modern and Pleistocene Large Herbivores

Analysis of the impact of water and food stress and other physiological factors



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Summary

Stable isotopes are a powerful tool for archaeologists and ecologists. However, the underlying factors that affect the stable isotope nitrogen ratios are still not known exactly. Especially, more detailed information is necessary about the influence that physiological adaptations to different environmental conditions have on the $\delta^{15}N$. Controlled feeding studies on living individuals might teach us more about the kind and measure of factors that manipulate the stable isotope ratios. Woolly mammoths show a remarkably high $^{15}N/^{14}N$ ratio in comparison with other contemporary living herbivores. Results in the present study show that modern elephants can be used to get insight in the mechanisms that determine the observed isotope values in fossil mammoths. Modern elephants could provide us with information about the influence that water and food stress have on $\delta^{15}N$ values. This is important, since results obtained in this study suggest that water and food stress might explain the enriched $\delta^{15}N$ values in woolly mammoths.

Contents

Introduction

Archaeology is the knowledge and study concerning material remains of the past. Archaeologists attempt to give an idea of the past in all its facets as accurately they can by analysing material culture.

One of the topics of interest in archaeology is food. Palaeodietary information of hominids can help us to gain an insight into aspects such as feeding habits, food processing, strategies concerning food-supply, nursing, food distribution, and economy, as well as ecological facets such as the environment and climate the hominids lived in (Schöninger *et al*. 1992, 249; Holden 2001, 403-11). Important information about such ecological aspects is also gained by analysing palaeodiets of animals, for most species have an explicit limit of tolerance concerning food-type, climate, and environment. But also information about aspects including weaning age, food preferences, health, and evolutionary changes can be derived from studies on the paleodiet of animals. An important advantage of paleodietary information provided by animals is that in archaeological sites animal remains are generally uncovered very frequently. Paleodietary reconstruction is interesting especially for extinct animal species, for not only does it give us an insight into their feeding habits, but it also provides us with information about their environmental circumstances; for example, the contemporary living plant and animal species, climatic conditions, and their level in the food chain (e.g. Bocherens 2003).

There are three sources commonly used for reconstructing the diet of an individual that lived in the past. The first are food remains preserved in, for instance, the stomach, intestinal tract, or faeces, which represent direct evidence of single meals. Ancient DNA research, palynology, and the study of plant macro remains are examples of methods used to detect and analyse food remains in such contents (e.g. Guthrie 1990; Van Geel 2008, 365, 366-70; Ukraintseva 1993; Holden 2001, 405-7).

Unfortunately, food remains are preserved only in extreme environmental conditions: in very hot, very dry, very cold or very wet circumstances. Therefore, well-preserved ancient food remains are extremely rare. Another problem is that certain food parts will be preserved, and therefore recognized, better than other parts. For example, easily digestible parts will be hardly discovered by studying macro remains, while these

might have played a chief role in the individual's diet. Moreover, several food types react differently to taphonomic processes and are less resistant to decay than others (Schöninger *et al*. 1991, 249-50; Holden 2001, 403-8; Mead *et al*. 1986, 121-2).

Other difficulties arise with regard to the short-term data on diet these food remains provide. The contents of the intestines give only an idea of the food an individual had eaten just before its death. Aspects such as cause of death (e.g. starvation or stomach disorder) and season of death have a strong influence on the nature of the individual's last meal (Holden 2001, 403-11; Van Geel *et al.* 2008, 365, 366-72; Guthrie 1990).

The second method used to recover the food pattern of an individual is by means of studying the morphology of its teeth, especially the molars. Generally, teeth survive very successfully, often even better than bones. The morphology of molars can provide us indirectly with information about the food habits of an individual. For example, the shape of a molar shows whether an individual was a carnivore, insectivore, omnivore, herbivore, or rodent (Marshall *et al.* 1965, 40-44).

Moreover, a strong correlation exists between hypsodonty, which is the crown-height of molars, and the kind of food consumed. Hypsodont animals have relatively high crowns and short roots. The tooth's crown height is determined by the distance between the biting surface and the starting point of the root's dentine. So, in life, this is the part between biting surface and gums. High crowns are well developed in mammals eating fibrous and abrasive food, for the molars of these animals are exposed to abrasion permanently. By developing higher crowns, the lifetime of the molar, and thus the animal, is longer (Olivier 1982, 293).

Additionally, microwear analysis on molars can give an indication about what an individual ate in the past. Analysis of dental microwear is the study of microscopic tooth-wear resulting from use. This method is based on the principle that hard, brittle foods leave a more complex microwear pattern than tough foods. When analysing dental microwear one should consider aspects such as jaw mechanics, biting function and age of the tooth, for these can cause differences in the microwear texture. One of the drawbacks of the dental methods mentioned above is that the morphology of molars only tells us about types of food eaten, but not about specific food products or amounts of food consumed.

The third source that provides insight into the feeding habits of humans and animals is stable isotopes of several elements in body tissues. The amount of certain stable isotopes is determined by the kind of food ingested. Depending on which stable isotope used, different kinds of information can be provided. For instance, the ratio $^{13}C/^{12}C$ ($\delta^{13}C$ value) can tell us about the plant types eaten, such as plants from temperate or tropical zones. The ratio $^{15}N/^{14}N$ ($\delta^{15}N$ value) can help us determine the trophic level of an individual, whether the individual lived in a marine or terrestrial environment, and in assessing weaning age. Because several food combinations can produce identical stable isotope values, stable isotopes are not suitable for identifying particular items of food (e.g. Schöninger *et al*. 1992, 248; Koch 2007, 129).

However, not only the kind of food and environment affect the isotope values. Other factors such as climate and physiology seem influence the stable isotope ratios as well (e.g. Ambrose 1991; Bocherens 2003; Fuller *et al*. 2005). The more factors are of influence, the less straight forward it is to draw conclusions about dietary information. Therefore, by extracting dietary information from stable isotope values, one should take into account as many factors of influence as possible.

Of course, ideally one should reckon with *all* factors that have an influence on the stable isotope values. However, finding out to which extent a certain factor played a role is very hard. This especially accounts for stable isotopes values derived from individuals that passed away long time ago. Controlled feeding studies on living individuals might teach us more about the kind and measure of factors that manipulate the stable isotope ratios. During such a controlled feeding study, the feeding pattern and the circumstances in life of an individual could be fully monitored during a certain period of time. Controlled-feeding studies are necessary to gain insight in processes of the stable isotope pathway and, therefore, to criticise the influence of different potential factors on the stable isotope ratio (e.g. Sponheimer *et al*. 2003c, 1653; Sponheimer *et al.* 2003a; Fuller *et al*. 2005 2505).

Woolly mammoths show a remarkably high $^{15}N/^{14}N$ ratio in comparison with other contemporary living herbivores. From evidence provided by their molars and from several studies on the intestinal tract contents of some woolly mammoths, we know that they were true herbivores. As a consequence, the high $\delta^{15}N$ values are caused probably by

factors other than just food (e.g. Bocherens 2003; Iacumin *et al.* 2000). We can consider what kind of factors these might have been, but, needless to say, a controlled feeding study would be impossible for woolly mammoths.

However, their modern relatives might be appropriate to be subject of a controlled feeding study. If modern elephants show comparable high $\delta^{15}N$ values relative to other herbivorous species, they might serve as a model for the Pleistocene woolly mammoths. To examine if the extremely elevated $\delta^{15}N$ values are typically for woolly mammoth or whether they are characteristic for elephantids in general, one should investigate if the pattern of the $\delta^{15}N$ values of Pleistocene herbivores is also present in their modern analogues. For that reason, this research compares the $\delta^{15}N$ values in bone collagen of mammoths, rhinoceros, and horse that lived on the mammoth steppe with the $\delta^{15}N$ values of modern elephant, rhinoceros and horse.

This thesis will focus on the high $\delta^{15}N$ values of woolly mammoths. The next chapter will provide a short outline of the use of different types of stable isotopes and their application in archaeology, whereupon the use of stable nitrogen isotopes in archaeology will be covered in more detail. The second part of this chapter will pursue the research questions this thesis deals with further.

In chapter three, the factors that might play a role in the elevated $\delta^{15}N$ values of Pleistocene mammoths will be considered.

The fourth chapter is subdivided in two sections. The first part contains information about the materials and methods used. Amongst others, the selection, extraction, the treatment of the samples, as well as information about the animals of which the samples are derived from (e.g. diet, age, and sex) will be expounded.

In the second section the results of the measurements- that is to say the stable isotope values of modern herbivores accomplished in Groningen- will be depicted. In Groningen, both $\delta^{13}C$ and $\delta^{15}N$ values of modern herbivores are measured. Although the focus will be on stable nitrogen, attention will be paid to the carbon results as well.

Finally, the results of the measurements will be discussed. The nitrogen and carbon isotope values will be compared with the isotope values of Pleistocene mammoth, rhinoceros, and horse. Moreover, all results will be examined regarding the materials and

methods used, and, moreover, these will be evaluated by virtue of the factors mentioned in the third chapter. In this final chapter, conclusions on the subject of this thesis will also be drawn.

Chapter 2 Application of stable isotopes analysis in archaeology and research topics of this thesis

2.1 Stable isotopes

Isotopes are atoms of the same element different in mass: they have the same number of protons and electrons, but they are distinguished by their different numbers of neutrons. Most elements have two or more isotopes that can be stable or unstable. Just a few of these elements have stable isotopes that are important to archaeological analysis: there are chiefly carbon (C), nitrogen (N), oxygen (O), hydrogen (H), sulfur (S), calcium (Ca), and strontium (Sr) (Schöninger *et al*. 1992, 253; Sulzman 2007, 1; Campbell *et al*. 2002, 28-9).

2.1.1 Principles

Several kinds of food leave behind different chemical characteristics in the consumer's body (McCutchan *et al*. 2003, 378-380). This is why Kohn (1999) stated that "You are what you eat" in his similar article. Stable isotope analysis is based on 'reading' these chemical signals (Kohn 1999, 335-6).

Depending on the kind of body tissue used as substrate for isotopic analysis, the stable isotope value provides information about a specific time slice. For instance, hair, feather, and nail keratin represent weeks or months, while in a sample of bone collagen several years of consumption are represented. The time represented by a sample depends on the tissue's turnover time and the sample strategy. While seasonality can be traced by studying hair samples, in bone samples both seasonal and longer-term variations are averaged. So, dependent on the kind of question one is dealing with, one should choose appropriate sample material (Gannes *et al*. 1998, 728-9).

Unfortunately, one is always dependent on the material available. Some tissue types easily decay, and, thus, very rarely recovered in archaeological contexts. Another issue one should keep in mind by choosing the sample material is the kind of element one wants to study: while carbon is present in almost all tissue types, strontium is available

only in bone bioapitite and tooth enamel (Schöninger *et al*. 1992, 248; Koch 2007, 100-4).

Moreover, different stable isotopes can provide different kind of information. For instance, while $\delta^{13}C$ can provide information about photosynthetic pathways, $\delta^{15}N$ can be used to determine an individual's trophic level. Thus, careful selection of the right element is very important. One should analyse the elements that are most suitable for the applications of interest (Koch 2007, 100-5; Rubenstein *et al*. 2004, 258).

2.1.2 Fractionation

Isotopic fractionation determines isotopic signals. This is a physical mass-dependent phenomenon causing changes in the relative abundance of isotopes due to their differences in mass. The difference in mass does not affect most aspects of chemical reactivity, but it can result in different rates of reaction between isotopes of a single element. This difference in rates often results in reaction products that have other isotopic compositions than that of the source material.

Because heavy atoms vibrate more slowly than lighter ones, the energy of molecules with heavier isotopes is lower, and they therefore form more stable, stronger bonds. The bonds containing the lighter isotope break and form more rapidly than bonds containing the heavier isotope during the transfer. These differences among isotopes lead to fractionations.

The degree of fractionation is typically quite small. A mass spectrometer is required for accurate detection of these small differences (Peterson *et al*. 1987, 294-300; Sealy 2001, 269; Sulzman 2007, 7; Schöninger *et al*. 1992, 253; Gannes *et al*. 1998, 726-8).

2.1.3 Method

A mass spectrometer is used to measure the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios. First, the (archaeological) material of interest is transformed to a gas: carbon is transformed to CO_2, ^{15}N is transformed to N_2. Then, a volume of CO_2 or N_2 is released into the mass spectrometer. Its electronic detector distinguishes between the stable isotopes of an

element. In this way, the isotope ratio of the sample's CO_2 and N_2 is measured. These amounts are compared to an internationally recognized laboratory standard. For carbon this is the marine PeeDee Belemnite Carbonate (PDB) or the more recently-determined Vienna PeeDee Belemnite (VPDB), and for nitrogen this is ambient inhalable reservoir (AIR).

These equations are noted in delta values (δ) in promille (‰), and are defined as indicated below, respectively for carbon and nitrogen:

$$\delta\,^{13}\mathrm{C} = \left(\frac{^{13}\mathrm{C}/^{12}\mathrm{C}_{\text{sample}}}{^{13}\mathrm{C}/^{12}\mathrm{C}_{\text{standard}}} - 1 \right) \times 1000\,‰$$

$$\delta\,^{15}\mathrm{N} = \left(\frac{^{15}\mathrm{N}/^{14}\mathrm{N}_{\text{sample}}}{^{15}\mathrm{N}/^{14}\mathrm{N}_{\text{standard}}} - 1 \right) \times 1000\,‰ .$$

A positive value indicates that the sample has more of the heavy isotopes than the standard. Most biological materials have a positive $\delta^{15}N$ value, for they have $^{15}N/^{14}N$ ratios that are higher than the atmosphere. Most biological materials have lower $^{13}C/^{12}C$ ratios relative to (V)PDB, and, therefore, these materials have a negative $\delta^{13}C$ value; a negative delta indicates the sample contains less of the heavy isotopes than the standard (Sulzman 2007, 6; Schöninger *et al*. 1992, 254 Gannes *et al*. 1998, 726).

2.1.4 Carbon

The stable isotopes of carbon ^{12}C and ^{13}C have a natural concentration in the atmosphere of 98.9% and 1.1% respectively (Sulzman 2007, 4). In the ocean, the amount of ^{13}C is slightly higher. The atmospheric CO_2 has a $\delta^{13}C$ values of about -8‰ (Bocherens 2003, 58, Marshall *et al*. 2007, 23), but plants contain less ^{13}C than the atmosphere in which they live, because of mass-dependent effects in the photosynthesis process. If plants take up CO_2 from the atmosphere by means of photosynthesis, they use relatively more ^{12}C than ^{13}C, which leads to a change in isotope ratio.

Three types of photosynthetic processes exist, used by C3-, C4- and CAM-plants (Marshall *et al*. 2007, 22-23). Plants that convert CO_2 to glucose by using products with three carbon atoms are called C3-plants. This kind of plant absorbs less ^{13}C in into their tissues than so-called C4-plants, which use connections of four carbon atoms. CAM-plants use either the C3- or the C4-photosynthetic pathway, depending on environmental conditions such as the amount of water and light available and the temperature. In general, trees, temperate grasses, and shrubs, are C3-plants. Tropical and savannah grasses, including maize and millet, are C4-plants. CAM-plants are typical succulent plants of the desert, including pineapples and cacti (Dincauze 2000, 365; Schöninger *et al*.1992, 255; Marshall *et al*. 2007, 24; Gannes *et al*. 1998, 727).

In contrast to most terrestrial plants, marine plants fix carbon photosyntatically different and have higher $^{13}C/^{12}C$ ratios (Schöninger *et al*.1992, 256).

The mean $\delta^{13}C$ value of C3-plants is about -26‰, and for C4-plants this is around -12‰. The $\delta^{13}C$ value of a plant is reflected in the plant consumer's bone collagen. The $\delta^{13}C$ values of herbivores are enriched by about 5‰ relative to the values of the plants consumed (Bocherens 2003, 59).

2.1.5 Nitrogen

In nature, ^{15}N and ^{14}N have a concentration of approximately 0.37% and 99.63% respectively (Sulzman 2007, 4). The $\delta^{15}N$ value is enriched by about 3‰ with each trophic level shift. The bone collagen of a consuming herbivore is enriched in ^{15}N relative to ^{14}N in comparison with the amount of ^{15}N originally in the plant available, and, on their turn, carnivores have higher $^{15}N/^{14}N$ ratios than herbivores (Schöninger *et al*. 1992, 258; Sealy 2001, 272; Richards *et al*. 2000, 7663; Gannes *et al*. 1998, 728-30).

As well as indicating the trophic level of an individual, $\delta^{15}N$ can tell us whether the food had been derived mainly from marine or terrestrial sources. Marine organisms on the base of the food chain generally have more positive $\delta^{15}N$ values than terrestrial plants. Vertebrates that live within a marine environment have higher $^{15}N/^{14}N$ ratios than terrestrial vertebrates. Thus, carnivores with a great deal of fish in their diets have higher $\delta^{15}N$ values than carnivores whose staple diet consists mainly of meat from terrestrial animals (Schöninger *et al*. 1992, 256-8).

Figure 1 shows schematically the general effects that several food sources have on stable carbon and nitrogen isotope values. The different photosynthetic pathways of plants consumed and marine food are probably the most important agents in determining the $\delta^{13}C$ values. An individual with a diet chiefly composed of C3-plants will have a lower $\delta^{13}C$ value than an individual with a staple diet of C4-plants. Somewhere in-between these values, the $\delta^{13}C$ values of herbivores with diets composed largely on CAM-plants will be situated.

Figure 1 also shows how $\delta^{15}N$ values increase as the trophic level is higher. Fish consumers show the highest $\delta^{15}N$ values. Moreover, consuming marine food contributes to higher $\delta^{13}C$ values as well.

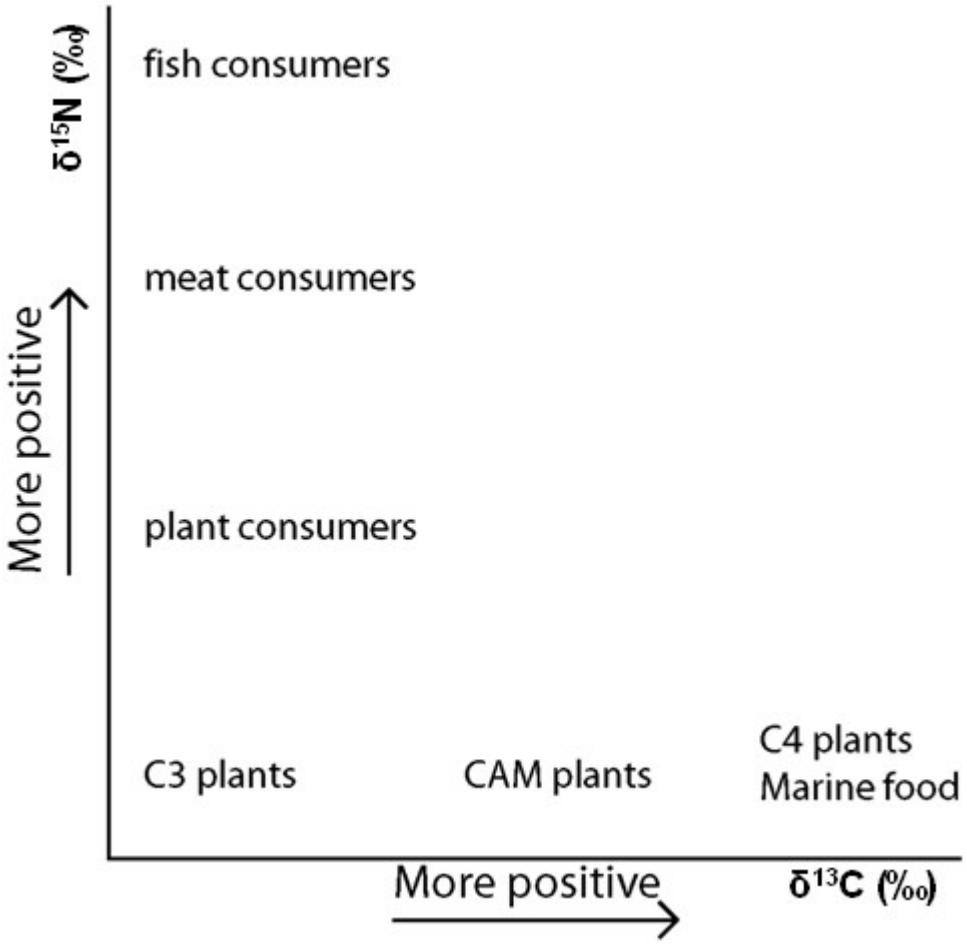

Figure 1 **Schematical view of the influence of food sources on isotopic values**

2.2 Research of this thesis

As stated above, it is generally accepted that the higher the trophic level of an animal, the higher the $\delta^{15}N$ value in the animal's bone collagen. Disciplines such as archaeology and ecology use $\delta^{15}N$ from body tissues increasingly as paleodietary indicators, assuming the $\delta^{15}N$ value to be elevated with about 3‰ with each tropic level shift (e.g. Sealy 2001, 272; Schöninger 1985, 516; Schöninger *et al*. 1992, 258; Richards *et al*. 2000, 7663). However, this is too simple a picture. In the next paragraphs the subject of this thesis will be discussed in more detail.

2.2.1 Values of Pleistocene species

If one were to apply this theory on $\delta^{15}N$ values of woolly mammoths, mammoths mistakenly would give the impression of being carnivores since their $\delta^{15}N$ values are extremely high in comparison with coeval herbivores (Bocherens 2003, 64-66; Kuitems 2008, 27-7) (Figure 2)[1]. This phenomenon does not form an exception in one geographical region of the mammoth steppe; a comparable pattern manifests in Europe (Bocherens *et al.* 1997), Siberia (Bocherens *et al.* 1996), and Alaska (Bocherens *et al*. 1994; Bocherens 2003) (Figure 3)[2].

Other examples of extraordinarily values are known, leading to wrong impressions: 'carnivore sheep' (Shishlina *et al*. 2007, 215-7), a vegetarian lion (Mol *et al*. 2009), Eskimos in Arabia (database CIO), and marine aurochs (Prummel *et al*. in prep.).

An overview of $\delta^{15}N$ values of Pleistocene mammoth, horse and rhinoceros, derived from the article from Bocherens (2003) and the database of the Centre for Isotope Investigation (CIO) in Groningen, is given in Appendix A. Table 1 shows that most values are derived from animals that lived in Europe. The majority of the mammoth values come from Siberia, and the majority of horses from Europe. From North-America

[1]Besides the strange values of the mammoth, the moose also shows remarkable values: see Figure 2. Ambrose stated in his article in 1991 that the low $\delta^{15}N$ values of moose might partly be explained by their large home ranges and access to water, for moose are water conserving species (Ambrose 1991, 298, 302, 309), but further study needs to be done on this topic.

[2] Figure 3 also shows a remarkable difference between the $\delta^{15}N$ values of mammoth bones from Alaska and Siberia. Although it would be interesting to find out what causes this variety, this is beyond the scope of this thesis. The research reported in this thesis is not hampered by the difference between the $\delta^{15}N$ values of Alaska and Siberia; the ratio between the δ15N values of mammoths and other herbivores is approximately the same in both Alaska and Siberia.

only the values of eight mammoths are used, and there was no information about horses in Bocherens' article or in the database of the CIO. The lack of rhinoceros values in North-America is plausible, since so far no remains from Pleistocene woolly rhinoceros have been found (Elias *et al*. 2008, 2473).

If we look at the average $\delta^{15}N$ values of all mammoths, rhinoceroses and horses, as compiled in Table 1, we can observe a shift of about 3‰ for mammoth in comparison with rhinoceros and horse. The discrepancy between mammoth and horse is a little larger than for mammoth and rhinoceros. Conversely, the $\delta^{13}C$ values are very similar, with mammoths having slightly increased $\delta^{13}C$ values in comparison to horses and rhinoceroses.

Table 1 Mean $\delta^{13}C$ and $\delta^{15}N$ values and standard deviations of Pleistocene mammoth, horse and rhinoceros derived from literature and the CIO database

	Animal	frequency	average $\delta^{13}C$	average $\delta^{15}N$	σ $\delta^{13}C$	σ $\delta^{15}N$
Total (97)	Mammoth	37	-21.61	8.74	0.59	1.66
	Rhinoceros	12	-20.74	6.08	0.62	1.31
	Horse	48	-20.11	5.62	0.68	1.47
Europe (57)	Mammoth	3	-21.33	8.7	0.38	0.61
	Rhinoceros	11	-20.75	5.87	0.65	1.14
	Horse	43	-20.99	5.61	0.64	1.52
Siberia (32)	Mammoth	26	-21.82	9.29	0.57	1.53
	Rhinoceros	1	-20.6	8.4	x	x
	Horse	5	-21.62	5.74	0.84	0.96
North-America (8)	Mammoth	8	-21.06	6.95	0.29	0.93
	Rhinoceros	0	x	x	x	x
	Horse	0	x	x	x	x

These values demonstrate that one must take into account that significant variation of $\delta^{15}N$ values can occur within a trophic level. Factors including climate,

environment and food source can cause considerable variation in $\delta^{15}N$ values of individuals from the same trophic level (Bearhop *et al*. 2004, 1009-12; Hedges *et al*. 2004, 962-4; Adams *et al.* 2000, 604-6; Ambrose 1991, 305).

Among others water stress, desert or saline soils, high grazing pressure, and marine environments cause relative high $\delta^{15}N$ values in plants. It is also known that grasses have higher nitrogen levels than shrubs, and even more so for trees (Bocherens 2003, 60; Sponheimer *et al*. 2003a, 81). Several studies of stomach contents of well preserved mammoths showed that the diet of mammoths consisted predominently of grassy material and that the mammoth steppe had a high grazing pressure. Therefore it is to be expected that mammoth bones show relative high $\delta^{15}N$ values. However, this applies not only to mammoths, but also to some other herbivores that lived on the mammoth steppe. The woolly rhinoceros (*Coelodonta antiquitatis*) and the horse (*Equus* species) lived under more or less the same circumstances (which implies having a comparable diet, living under the same weather conditions and all being monogastric). Nevertheless, the $\delta^{15}N$ values of the woolly rhinoceros and the horse are considerably lower than these of the mammoth (Figure 2) (Bocherens 2003). In Appendix B an extended overview is provided on the diets of these Pleistocene animals.

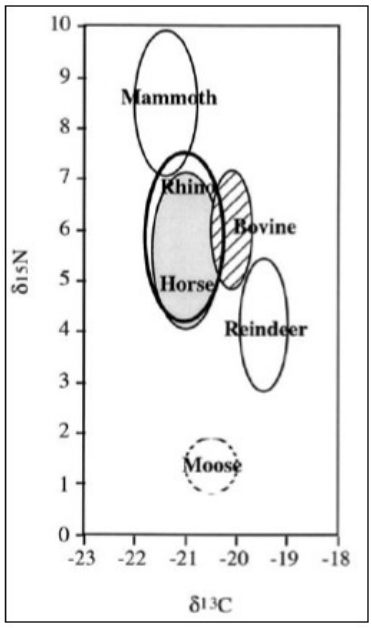

Figure 2 Average and standard-deviation of $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen of mammoth steppe herbivores. (From: Bocherens 2003, 66)

It is important to improve our understanding of (zoo)archaeological $\delta^{15}N$ data in general. In particular, the difference between the $\delta^{15}N$ values of woolly mammoth and those of woolly rhinoceros and horse needs to be explained. Apparently, one needs to examine factors other than diet, environment and climate that possibly influence $\delta^{15}N$ values. Among others, the influence of physiological dynamics on $\delta^{15}N$ values might be assessed in order to understand the interspecific variation in the nitrogen isotopic composition of herbivore bone collagen within habitats (e.g. Schöninger *et al*. 1992, 282; Sealy 2001, 272; Ambrose 1991, 294).

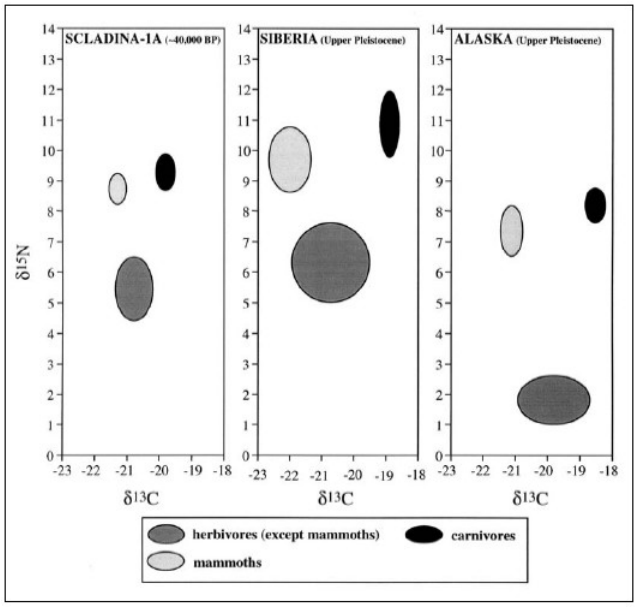

Figure 3 Carbon and nitrogen isotopic variations in Upper Pleistocene mammal collagen from Siberia, Alaska and Scladina (average ± one standard-deviation). (From: Bocherens 2003, 65)

2.2.2 Aim of this research

Several studies have been performed on stable isotope values of Pleistocene mammals, including $\delta^{15}N$ values of mammoths (e.g. Bocherens *et al*. 1996, 1997; Bocherens 2003; Coltrain *et al*. 2004; Iacumin *et al*. 2000). However, the underlying cause or causes that affect the high values of mammoths are still not known exactly, because an appropriate approach of analysing $\delta^{15}N$ values has yet to be established. Known factors that cause increased $\delta^{15}N$ values in bone collagen do not seem to explain the high values observed in mammoth bones.

Former investigations on variation in nitrogen isotope composition in bone collagen occurring within one trophic level and within one habitat suggested several possible factors. Before establishing which factors might cause the elevated $\delta^{15}N$ values in mammoth bone collagen, one should first examine as many potential factors as possible to cover this broad field of research. Therefore this thesis is first of all aimed at giving an overview of the most important of such possible factors.

Controlled-feeding studies are necessary to get insight in processes in the nitrogen pathway, enabling the evaluation of different potential factors (Sponheimer *et al*. 2003c, 1653). It is obviously impossible to perform a controlled-feeding study on woolly mammoths, woolly rhinoceros and coeval horses, for they died out a long time ago. The closest thing to such a study on these mammoth steppe animals is a controlled feeding study on extant relatives of these animals, such as the modern elephant, rhinoceros and horse.

To investigate whether the elevated $\delta^{15}N$ values are typical for woolly mammoths, or whether they are characteristic for elephantids in general, one might find out if the pattern of the $\delta^{15}N$ values of Pleistocene herbivores is also present in their modern analogs. Figure 4 presents two potential patterns which might be derived from the results of this research. The left graph demonstrates a pattern in which the $\delta^{15}N$ values of elephant, horse, and rhinoceros are more or less equal. In this case, the modern elephant does not form a suitable model for Pleistocene mammoths regarding the stable nitrogen isotope value. The graph on the right shows a pattern in which the $\delta^{15}N$ values of elephants are clearly enriched relative to these of horse and rhinoceros. If such a pattern were to obtained, elephants' $\delta^{15}N$ values would behave roughly the same as these of Pleistocene mammoths, which implies that modern elephants could serve as models for mammoths during controlled feeding studies.

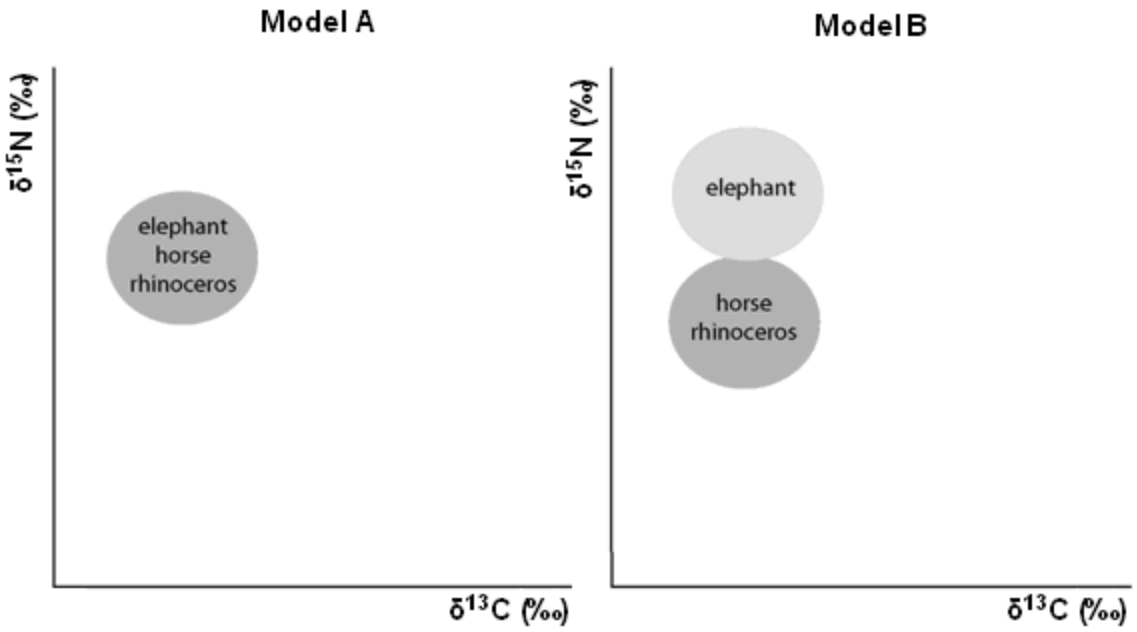

Figure 4 Schematical reflection of two potential outcomings of the results derived from this research

Thus, this research compares the $\delta^{15}N$ values in bone collagen of mammoths, rhinoceros, and horse that lived on the mammoth steppe with the $\delta^{15}N$ values in hoof and nail material of modern elephant, rhinoceros and horse, and, the results will be evaluated by means of factors presented in chapter 3. To be precise, the specific research questions in this thesis are:

- *Which factors might play a role in determining the $\delta^{15}N$ values of mammoths?*
- *Could the modern elephant, rhinoceros and horse, in controlled feeding studies serve as model for Pleistocene mammoth, rhinoceros and horse to enlighten the factors that affect their stable isotopes values?*

Chapter 3 Factors that might play a role in defining the stable isotope values of herbivores

3.1 Introduction

As discussed in the previous two chapters, isotopic data needs to be processed in a different manner from traditional methods: more specifically, one should not simply assume that the $\delta^{15}N$ value will be elevated by about 3‰ with each trophic level shift. Unfortunately, an appropriate alternative approach of analysing $\delta^{15}N$ values has yet to be presented, since the underlying cause or causes that affect the high values of mammoths have not yet been established. There are many recognized factors that could cause increased $\delta^{15}N$ values in bone collagen, but so far they have not been exhaustively considered in relation to the high values found in mammoths.

To clarify the elevation of $\delta^{15}N$ values in mammoths, first of all a suitable approach for analysing $\delta^{15}N$ values in general is needed. We have to understand the basics of diet-tissue isotope fractionation and we need to know more details of the possible influences that several factors have on mammalian nitrogen isotope compositions to make the most of the information that $\delta^{15}N$ values offer. Therefore, in this chapter, the factors that might play a role in the elevated $\delta^{15}N$ values of mammoths will be considered. First of all, the natural nitrogen pathway, 'the surroundings' shall be examined, and variation in its course under different conditions shall be explored. Consequently, a simple overview of the general nitrogen cycle inside the body will be provided, together with the nitrogen cycle in the surroundings, thus a foundation for the more complicated topics that follow.

Accordingly, we discuss the nitrogen trail within the organism in more detail, and study the related physiological processes and adaptations to different environmental circumstances and their effects on the $^{15}N/^{14}N$ ratio. These are physiological adaptations, mainly concerning metabolism, osmoregulation, and digestion. Although in certain environmental circumstances one specific physiological aspect seems to be key agent in defining the nitrogen isotope ratio, generally different aspects are interrelated. In this

chapter, some important physiological aspects will be discussed by means of the black box system.

Finally, physiological processes in themselves which are not affected by the environment will be discussed in the light of their potential influence on the nitrogen isotope ratio. Age, sex and pregnancy are examples of such processes.

3.2 Nitrogen pathway outside the body

Nitrogen is the most abundant element in the atmosphere (about 80%). It is mainly available as gas (N_2). This nitrogen pool is well-mixed with an isotopic composition that is constant at 0‰. This N_2 gas must be converted to ammonium (NH_4^+) or nitrate (NO_3^-) before it can be used and absorbed by plants. Plants have great need for nitrogen, because it forms a crucial component of the nucleic acids DNA and RNA, proteins and many vitamins. Animals obtain nitrogen by consuming plants. This process is shown in Figure 5.

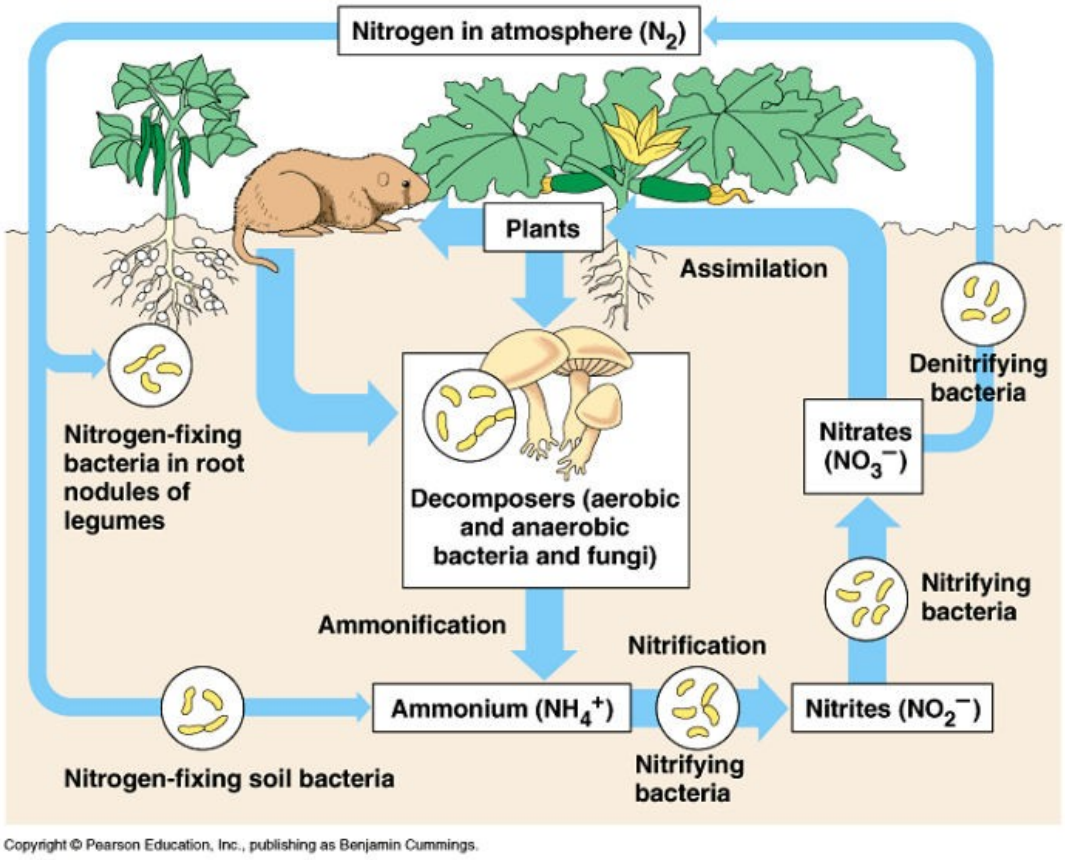

Figure 5 The nitrogen cycle (From: Campbell 2002, 1211)

Figure 5 shows the processes nitrogen undergoes from atmospheric N_2, via soil to plants, and finally into N_2 gas again. Generally, nitrogen is made accessible to organisms by nitrogen fixation or bacterial breakdown. However, a nitrogen source not mentioned in Figure 5 is 'atmospheric deposition'. In this process, NH_4^+ and NO_3^- are added to soil after being dissolved in precipitation.

Nitrogen fixation is executed by N_2-fixing bacteria, in both marine and freshwater circumstances, in the soil, and on plant roots in terrestrial environments. Bacteria in the soil can convert N_2 into compounds useful as nutrients for plants and animals by nitrogen fixation, where N_2 is converted to NH_3 (ammonia). Aerobic bacteria can convert ammonia not taken up by plants into nitrate ions (NO_3^-) by means of nitrification. These nitrate ions can easily be taken up by plants as a nutrient. Plant roots can absorb ammomium ions (NH_4^+), which are dissolved to the soil water by the aforementioned nitrogen fixation and nitrification processes.

Another source of nitrogen is dead organisms or excretions by means of bacterial breakdown. Bacteria and fungi can convert these 'nitrogenous wastes' into NH3 and NH_4^+; this process is called ammonification. Anaerobic bacteria can convert NH_3 and NH_4^+ into nitrogen gas (N_2) and nitrous oxide gas (N_2O), which are released into the atmosphere (Miller 2005, 80-1; Campbell 2002, 774-5, 1210-1; Schöninger *et al*. 1992, 256; Peterson *et al*. 1987, 302).

The nitrogen fixation process results in synthesized tissues with $^{15}N/^{14}N$ ratios similar to atmospheric N_2 (about 0‰). The $\delta^{15}N$ values of the nitrates derived from bacterial breakdown contain more ^{15}N relative to ^{14}N than is true in the atmosphere. Therefore, the plants using these nitrates derived from bacterial breakdown tend to have slightly more positive $\delta^{15}N$ values than N_2-fixing plants. Plants' nitrogen is for the most part the result of nitrogen-fixation processes, while nitrogen derived from bacterial breakdown makes a smaller contribution to plants. For this reason, most terrestrial plants have values similar to atmospheric N_2 (Schöninger *et al*. 1992, 256).

Several factors influence the $\delta^{15}N$ values of plants (e.g. Ambrose 1991, 296-7; Marshall *et al.* 2007, 22-50). However, to give an overview of these factors is beyond the scope of this thesis.

3.3 Nitrogen pathway inside the body

3.3.1 Introduction

As mentioned above, nitrogen is a constitutional chemical element of the nucleic acids DNA and RNA, and proteins. Moreover, nitrogen plays a role in excretion, acid-base regulation, and in osmoregulation. Therefore, to survive, animals must use nitrogen-containing amino acids from plant sources as starting materials for metabolizing nitrogen, and for the manufacture of proteins and nucleic acids.

Animals take in nutrients and excrete waste products. One can subtract nitrogen output from nitrogen input to estimate the number of nitrogen fractions retained in the body and used to synthesize tissues:

NITROGEN IN TISSUES=NITROGEN INGESTED − NITROGEN EXCRETED

This is the idea of the 'nitrogen isotope mass balance model': if one knows the composition of nitrogen ingested and that of the nitrogen excreted, one can calculate approximately the amount of nitrogen that is retained in the body.

Normally, well-fed, healthy, adult animals are in nitrogen balance, where nitrogen ingested equals nitrogen excreted. However, certain factors effect the nitrogen balance, causing a negative or positive nitrogen balance. In case of a negative nitrogen balance, the amount of nitrogen excreted from the body is greater than the amount of nitrogen ingested. A nitrogen intake higher than the loss of nitrogen from the body results in a positive nitrogen balance.

Figure 6 shows a simple black box system. We should consider the mammoth as such a black box to investigate the influence of diverse potential factors. The black box does not stand on its own, but it is constantly in interaction with its surroundings. Namely, the input of the black box is derived from the environment and the output, in turn, is directly deposited in its surroundings.

Animals must always adapt to environmental situations. These might include climatologically and milieu concerning circumstances. For instance, adaptations that reduce water loss are necessary for survival on land; this is why most terrestrial animals

have physiological mechanisms which help prevent dehydration (Campbell *et al*. 2002, 941). Next, several of these adaptations and their potential effects on the $\delta^{15}N$ values will be discussed.

Figure 6 Black box system

3.3.2 Osmoregulation

Unlike plants, animals cannot store amino acids to any great extent in their tissues. Much of the nitrogenous matter secreted into the gut lumen is itself subject to digestion and reabsorption, so only a small fraction of the total nitrogen secreted into the lumen is lost in the feces (Fuller *et al*. 1998, 386; Singer 2003, 543).

Deamination is the process in the liver by which excess amino acids are broken down and the amino group converted to ammonia. One major problem that is that ammonia, NH_3, is very toxic (Wright 1995, 274; Marshall *et al*. 1965, 135). Hence the animal should get rid of such waste products, before they accumulate to toxic levels (Wright 1995, 273; Bentley 1971, 34). A major factor in determining the mode of nitrogen excretion is the availability of water in the environment, for most metabolic wastes must be dissolved in water when they are removed from the body (Wright 1995, 273).

Fortunately, an organism can control the amount of its waste products and body water by means of osmoregulation, by which it can harmonize the uptake and loss of body water and solutes.

Animals excrete a variety of nitrogen waste products. Depending on their type and quantity, waste products can have various effects on an organism's osmoregulation. Nitrogen-containing breakdown products of proteins and nucleic acids are very common

wastes. Enzymes remove nitrogen in the form of ammonia, which although soluble, can only be tolerated at very low concentrations. This is why animals that excrete nitrogenous wastes directly as ammonia need access to lots of water.

Whereas aquatic species can excrete ammonia directly, most terrestrial animals simply do not have access to sufficient water (Bentley 1971, 34). During the evolution of terrestrial animals, water conservation became an important concern. Therefore, most terrestrial mammals convert ammonia to urea, a compound that can be concentrated in body fluids to a greater extent than ammonia, and with no toxic effect. Urea requires about 10 times less water than ammonia for excretion. A tiny fraction of the urea synthesized is cycled to the gastrointestinal tract, while the remainder is excreted in the urine (Singer 2003, 543-4).

The main disadvantage of urea is that animals must expend energy to produce it from ammonia. This production occurs in the vertebrate liver by a metabolic cycle (the urea cycle or ornithine cycle), in which ammonia is combined with carbon dioxide. Finally, urea is transported to the excretory organs, the kidneys (Campbell *et al*. 2002, 937).

Water stress: urine concentration

Several investigations have shown that, within ecosystems, herbivore species with physiological adaptations to maintain water have higher $\delta^{15}N$ values than water-dependent species. Ambrose and DeNiro stated in 1986 that 'Species described as drought-tolerant or as having the ability to survive for long or indefinite periods without drinking water have mean $\delta^{15}N$ values that are 2-4 per mil higher than those species collected in the same environments that are classified as obligate drinkers' (Ambrose *et al*. 1986, 398).

An explanation for these physiological adaptations is that changes in rates of urea excretion in response to water stress have an effect upon nitrogen isotope mass balance. The amount of urea in urine can be regulated through osmolality (the concentration of an osmotic solution). During periods of water shortage, when individual need to save body water, urea is present in urine in a highly concentrated form: water stress leads to increased urea nitrogen loss. The maximum urine-concentrating capabilities of mammals

vary enormously. In order to maintain nitrogen balance, the animal needs to eat a high-protein diet. During the dry season, the protein content of most leaves is higher relative to that of dry grass. Therefore, browsing animals generally consume more protein than grazers during periods of drought, and are more able to supply urea to the kidneys when water-stressed. In addition, generally browsers are able to concentrate their urine to a greater degree than grazers, and are therefore more drought tolerant (Sealy *et al*. 1987, 2708; Ambrose 1991, 305-10; Iacumin *et al*. 2000, 34).

Given that urea is the major form of excreted nitrogen in mammals, this could cause changes in the nitrogen isotope composition in excretion, and therefore in tissues (Ambrose 1991, 305-10). Figure 7 shows that the higher the urine osmolality, the higher the $\delta^{15}N$ values of animals.

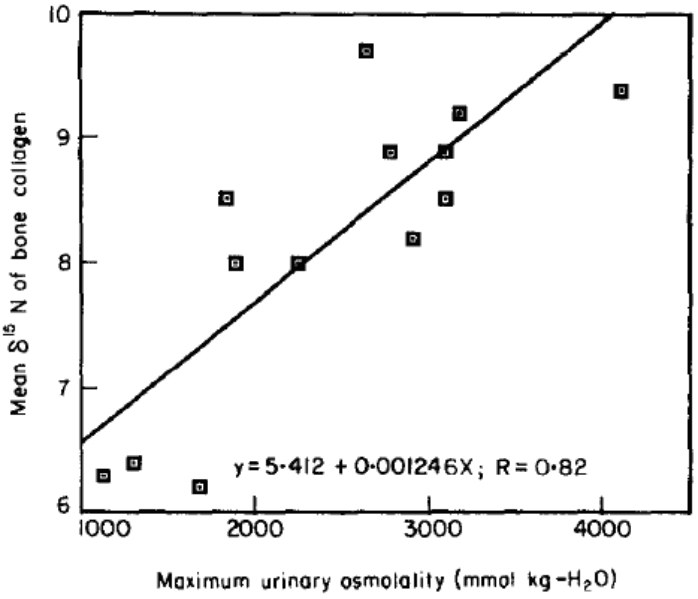

Figure 7 The relationship between maximal urinary osmolality and mean $\delta^{15}N$ values derived from bone and tooth collagen of 13 modern east African herbivores (From: Ambrose 1991, 309)

Although there is some debate about the precise mode of fractionation, the chemical basis for this fractionation is that heavier isotopes form bonds of greater energy than their isotopically lighter counterpart and thus are less likely to undergo chemical reactions (Gannes *et al*. 1998, 728; Adams *et al*. 2000, 601). Physiologically, this implies that amine groups containing ^{14}N are favored during transamination and deamination, so

that more $\delta^{15}N$ is incorporated into collagen and that more of the isotopically lighter ^{14}N is excreted (Figure 8) (Ambrose 1991, 305; Schöninger *et al*., 1984; Adams *et al*. 2000, 601). Consequently, excreted nitrogen (i.e., ammonia, uric acid, or urea) is ^{14}N-enriched relative to animal protein (Gannes *et al*. 1998, 728; Adams *et al*. 2000, 601).

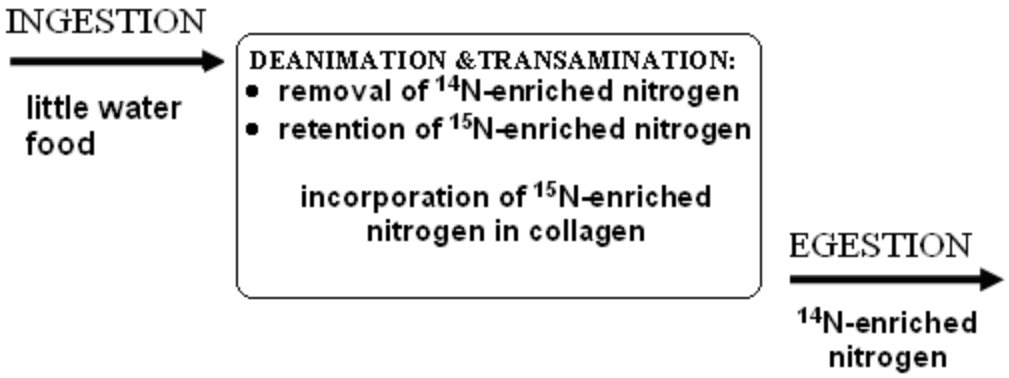

Figure 8 The effect of water stress on stable nitrogen isotope ratios

Water and food stress: nitrogen recycling

Rather than increased rates of urea excretion under circumstances of water stress, there would be a dramatic reduction among animals on nitrogen-poor diets (Singer 2003, 548; Martin 1982, 263). Such animals could not afford to excrete urea freely; instead, it has to be recycled to maintain a suitable substrate for the herbivore's gut flora (more about the role of the gut flora will be mentioned in paragraphs below).

Evidence is accumulating that suggests isotopic fractionation across trophic levels may depend on nitrogen availability. As indicated before, increases in $\delta^{15}N$ values of animals occur due to preferential loss of ^{14}N. Moreover, ^{14}N loss has been shown to increase during periods of both water and nutritional stress, for the concentration of excreted urea in mammals increases under water stress. During starvation nitrogen uptake is near zero, but nitrogen loss remains, even if at a low rate. Unfed animals have been found to show increasing $\delta^{15}N$ values due to the recycling of existing nitrogen (Hobson *et al*. 1993, 390-2; Adams *et al*. 2000, 605).

As available nitrogen decreases, organisms are forced to rely more heavily on internal nitrogenous resources. The preferential excretion of ^{14}N from this internal

nitrogen reserve may result in increased organismal $\delta^{15}N$ values with decreased nitrogen availability (Adams *et al*. 2000, 605).

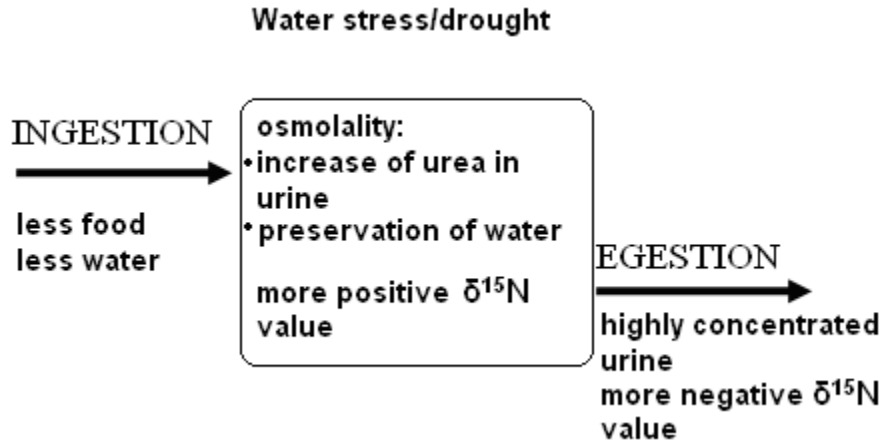

Figure 9 Protein or energy deficiency results in breakdown of the body's own proteins. The mammals respond to a decreased nitrogen intake by reducing the rates of urea synthesis and urinary urea excretion (Singer 2003, 548). For this reason, in periods of fasting or starvation there is a negative nitrogen balance, which means that the amount of nitrogen excreted from the body is greater than the amount of nitrogen ingested. Meanwhile, due to recycling, the pool of nitrogen that is not excreted and is available for tissue synthesis must have significantly more ^{15}N than the diet.

Figure 9 shows that under stress, when animals excrete more ^{15}N-depleted urea, and eat less food, the pool of nitrogen that is not excreted and is available for tissue synthesis must have significantly more ^{15}N than the diet. Conversely, in unstressed animals excreting less ^{15}N-depleted urea, while eating more food, the pool of nitrogen available for tissue synthesis must have significantly less ^{15}N (Figure 10).

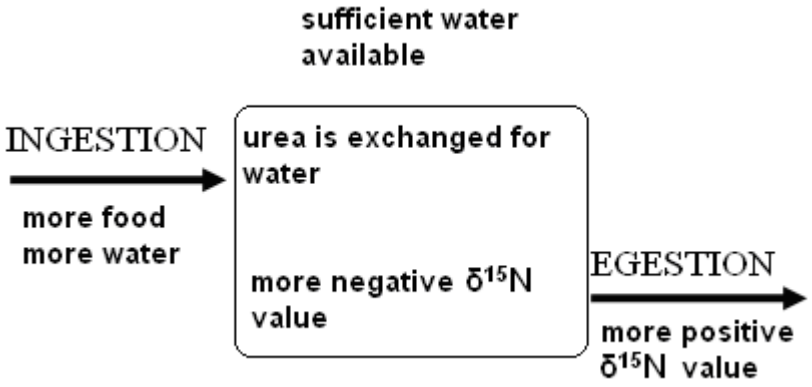

Figure 10 If food and water is sufficient, an organism does not need to concentrate its urine. This leads to 'normal' stable nitrogen isotope ratios

Browsing and grazing

Summarising, one might conclude that mammals that can excrete highly concentrated urine (mostly browsers or mixed feeders) should have more positive $\delta^{15}N$ values than those that cannot (mainly grazers): the highest $\delta^{15}N$ values are correlated with the highest osmolalities. Individuals from hotter, more arid environments should have more positive tissue $\delta^{15}N$ values than these of the same species from cooler, humid ones (Ambrose 1991, 305-10).

However, there are data available which show that the contrary might also be the case. Research performed by Sealy and his colleagues in 1987 on stable nitrogen isotope ratios of grazers, mixed feeders, and browsers from the Addo National Park in the Eastern Cape (an area with less than 400 mm of rain), showed that the browsing species have slightly lower $\delta^{15}N$ values than the grazers (Sealy *et al*. 1987, 2713).

One of the reasons for this result might be that not all grazers are obligate drinkers, nor all browsers drought-tolerant and urine-concentrators; it is true that some grazers can excrete more concentrated urine than closely-related browsers.

Moreover, Sealy and his colleagues (1987) suggested that differences in the recycling of urea might have led to differences in the tissue $\delta^{15}N$ values: 'Animals in areas receiving less than 400 mm of rain per annum may consume rather low-protein diets and hence be more dependent on recycling their urea to conserve nitrogen. If this is the case, and grazers habitually consume diets lower in protein than browsers, then grazers might be expected to recycle their urea to a greater extent than browsers. If each cycle increases the $\delta^{15}N$ of the protein synthesized by the symbiotic microorganisms, then grazers from arid areas should have higher $\delta^{15}N$ values than browsers. This process may explain the patterning in the Addo and Lake Turkana samples (Sealy *et al*. 1987, 2714).

However, Ambrose (1991), stated that the model proposed by Sealy *et al*. (1987) 'will not account for higher $\delta^{15}N$ values among grazers than browsers from the same ecosystem' (Ambrose 1991, 310). According to Ambrose, rather than leading to an enrichment of $\delta^{15}N$ values in tissues, the model of Sealy would lead to depletion.

Therefore, another model needs to be developed to explain the increased $\delta^{15}N$ values in bone collagen of grazers from the Addo National Park. The protein content of the diet might be a relevant variable effecting increased $\delta^{15}N$ values (Ambrose 1991,

310). The influence of the protein level of a diet on the nitrogen isotope ratio will be discussed further in the following section.

3.3.3 Digestion

Digestion is the process whereby the various items of the diet are broken down into molecules small enough to be assimilated into the blood stream or lymph system. In all mammals digestion takes place in multiple stages, starting with ingestion and ending with egestion (Figure 11).

Ingestion is the intake of food. By chewing food in the mouth, mammals break it down mechanically ('mechanical breakdown'). The food is further broken down in the buccal cavity by salivary enzymes, and in the stomach and intestines by bile, acids and enzymes ('chemical breakdown'). By this stage, the nutrients in the digestive system are small enough to be absorbed. Finally, the matter which remains undigested and unabsorbed is removed from the digestive tract by excretion (Marshall *et al*. 1965, 20-1; Campbell *et al*. 2002, 857).

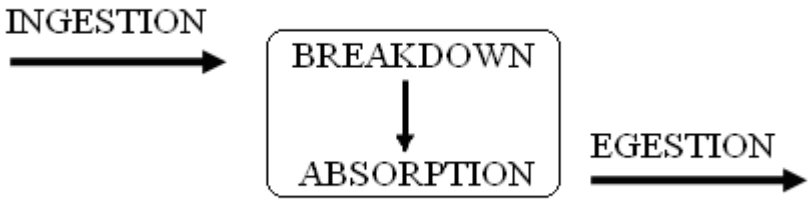

Figure 11 Digestion

While Figure 11 is representative for all mammals, differences within the organ structures and eating behaviours exist as a result of adaptations to particular dietary components and availabilities of nutrients within a particular diet. For instance, generally herbivores and omnivores have longer alimentary canals relative to their body size than carnivores, for vegetation is more difficult to digest than meat because it contains cell walls. A longer tract gives more time for digestion and more surface area for the absorption of nutrients (Campbell *et al*. 2002, 867; Olivier 1982, 293).

Herbivorous animals face a special challenge: much of the chemical energy in their diets is contained in the cellulose plant cell walls, but animals do not produce enzymes that hydrolyse (break down) cellulose. Many vertebrates solve this problem by housing large populations of symbiotic bacteria in special fermentation chambers in their alimentary canals. These micro organisms *do* have enzymes that can hydrolyse cellulose to simple sugars and a variety of nutrients that are essential for the animal, such as vitamins and amino acids. Hence, the micro organisms the animal to eat a wider range of food stuffs that would not be digestible without their aid (Marshall *et al.* 1965, 44; Olivier 1982, 292).

Both hindgut and foregut fermenters rely on the production of these nutrients by bacteria to derive their dietary energy. While some hindgut fermenters rely almost exclusively on bacterial processing of forage diets, others derive only a portion of their energy needs from this process. The degree of reliance on hindgut fermentation typically depends on diet. The main difference between foregut fermenters and hindgut fermenters is that the latter have a relatively simple, small undivided stomach, but a larger cecum and colon where the microbes are housed and where fermentation takes place (Olivier 1982, 292-4).

These variations in digestive structures can cause different $\delta^{15}N$ values amongst species. Therefore it is important to consider the influence of digestion on the nitrogen isotope ratio. Hence, the next paragraph will focus on digestive systems in relation to their potential influence on the nitrogen isotope ratio.

Adaptions to disadvantages of hindgut fermentation

An advantage for animals that utilise cecal fermentation is that if they have to deal with low protein diets, they are able to increase food intake obtain sufficient nutrients (Olivier 1982, 293).

However, in general, hindgut fermenters are at a slight disadvantage compared to foregut fermenters because in hindgut fermenters the food passes first through the main absorptive region of the gut (small intestine), before fermentation takes place by the microbes in the cecum and colon. Therefore, nourishing by-products of fermentation by bacteria are initially lost with the feces (Campbell *et al*. 2002, 868).

Animals developed different adaptations to improve their rates of digestion and absorption. One of these adaptations is 'intestinal reflux', which is used, amongst others, by horses. It makes food move back into the small intestine by reverse peristaltic movements.

Animals that do not apply this intestinal reflux face the problem that much of the protein (and some vitamins) that are produced by the fermenting bacteria is lost through the feces. Some hindgut fermenters such as rabbits and elephants solve this problem by practicing coprophagy. After excretion, droppings existing of partly digested food are immediately eaten again, so the food material can pass through the digestive system a second time. By doing so, they capture nitrogen, mostly in the form of protein and free amino acids, that would have otherwise been lost. Recently, it has been shown that mammoths also practiced coprophagy (Van Geel *et al*. 2008, 369).

Elephants, horses and zebras, and rhinoceroses are monogastric hindgut fermenters with large cecums (Sponheimer *et al*. 2003a, 82; Clauss *et al*. 2003, 161; Martin 1982, 259). It has been suggested that digestive anatomy plays a role in determining herbivore $\delta^{15}N$ values. According to Sponheimer and his colleagues, foregut fermenters should have higher $\delta^{15}N$ values relative to hindgut fermenters (Sponheimer *et al*. 2003a, 81).

3.3.4 Reproduction

Several studies have investigated the potential rate of influence on stable nitrogen isotope ratio of aspects concerning reproductive biology. Here, the role of pregnancy, suckling, and lactation within $\delta^{15}N$ values will be discussed.

Lactating mothers catabolise their own tissues to produce milk. The nitrogen accessible in the child's milk has the same $\delta^{15}N$ value as that existing in the tissues of its mother. In fact, the suckling child is consuming its mother, so, relative to its mother, one would expect that its $\delta^{15}N$ value looks like the offspring is feeding at a higher trophic level with an enrichment of about 3‰ (Koch 2007, 129; Fogel *et al*. 1997, 278; Jenkins *et al*. 2001, 336).

While Fogel and his colleagues stated in 1997 that lactating women do not show an isotopic effect as a result of the breastfeeding (Fogel *et al*. 1997), evidence is

accumulating that pregnant females have lower $\delta^{15}N$ values in comparison to other adults (Koch 2007, 131; Fuller *et al*. 2004, 2889-2895; Fuller *et al*. 2005, 2497). Fuller and colleagues (2004) showed a decrease in hair $\delta^{15}N$ values for several pregnant women, with $\delta^{15}N$ values decreasing from conception to birth. The reason for this decrease in hair $\delta^{15}N$ during pregnancy is still unknown, but an increase of nitrogen retention during gestation might play a role (Fuller *et al.* 2004, 2894).

So far, we do not know whether this isotopic depletion observed in hair can be seen in bone collagen, since it continuously remodels; however, other tissues such as nails, teeth, feathers, horns and tusks suggest the possibility of detecting pregnancy (Fuller *et al.* 2004, 2895). However, one should keep in mind that periods of morning sickness generally cause increased $\delta^{15}N$ values due to weight loss or restricted weight gain (Fuller *et al*. 2005, 2497). Namely, to support the growth of the mother and the foetus, a pregnant female should consume more protein per day than usual. The restricted nutrient intake during period of morning sickness necessitates the female to rely on her own tissues as her nitrogen source (Fuller *et al*. 2005, 2503-4). This implies that the excreted lighter nitrogen (^{14}N) is not replaced by dietary protein. This is why the body tissues become progressively enriched in heavier nitrogen (^{15}N) as the duration of the morning sickness progresses (Fuller *et al*. 2005, 2504).

Thus, regarding the nitrogen balance, pregnant individuals have a positive nitrogen balance. Their nitrogen is higher than normal and also higher than the loss of nitrogen from the body and, at the same time, pregnancy causes depleted $\delta^{15}N$ values. Morning sickness is a catabolic state resulting in an increase of the individual's $\delta^{15}N$ values, but in a negative nitrogen balance (Fuller *et al*. 2005, 2503; Koch 2007, 109).

3.3.5 Age

It has been suggested that age influences isotope ratios (Dalerum *et al*. 2005, 653-4). Most studies, however, do not show a differential age effect on diet to tissue fractionation for either $\delta^{13}C$ or $\delta^{15}N$ (Jenkins *et al*. 2001, 339; Schwarz 1991, 268; Sponheimer *et al*. 2003a, 83). Amongst others, Minagawa and Wada (1984) investigated the relationship between the nitrogen isotope ratio of an animal and its age on two kinds of marine mussels with identified age (Minagawa *et al*. 1984, 1135). They concluded that the $\delta^{15}N$

value of marine mussels is independent of both their age and the nitrogen mass balance in the body (Minagawa *et al*. 1984, 1137).

Moreover, in 2006 Nardoto and his colleagues performed a study on $\delta^{13}C$ and $\delta^{15}N$ values of human fingernails derived from individuals from the western United States of America and southeastern Brazil. They observed no significant isotope ratio dependence on the age of individuals (Nardoto *et al*. 2006, 137-9).

Furthermore, a mathematically simple model of the trophic isotopic enrichment phenomenon in animals developed by Ponsard *et al.* (1999) showed that growing mice and rats should show a $\delta^{15}N$ similar to that of the adult (Ponsard *et al*. 1999, 1305-9).
On the other hand, some other studies demonstrated that $\delta^{15}N$ values can vary considerably according to age (Caut *et al*. 2008 256). For instance, Roth and Hobson (2000) found a very small but significant degree of difference on $\delta^{15}N$ values between tissues of subadult and adult red foxes (*Vulpes vulpes*). Foxes younger than 1 year old were enriched in ^{15}N compared with adult foxes. A study on water fleas by Adams *et al*. (2000) showed that animals with higher $\delta^{15}N$ values were older (Roth *et al*. 2000, 848; Adams *et al*. 2000, 605).

Neither the investigators of the Daphnids nor of the foxes were able to explain the precise mechanism that the caused this enrichment. Dalerum *et al*. (2005) and Roth *et al*. (2000) stated that metabolic pathways during times of growth might play a role in stable nitrogen isotopic change in relation to age (Roth *et al*. 2000, 848; Dalerum *et al*. 2005, 653-4). Rapidly growing children have a higher rate of protein synthesis than mature adults, which should also lead to a more evident influence of a particularly dietary source in the children's body tissues (Fogel *et al*. 1997, 280).

3.3.6 Sex

Several investigations have been performed to find out if differences in sex have any influence on the stable isotope values. DeNiro and his colleagues (1983) suggested that there is no significant difference in the isotopic values in bone collagen between males and females for either nitrogen or carbon (DeNiro *et al.* 1983, 201-2).
Also, Schwarz stated in 1991 that the isotopic ratios of bone collagen from males and females feeding on the same diet are identical. They assume that any difference in

nitrogen or carbon values associated with age or sex is due to difference in diet (Schwarz 1991, 268).

Chapter 4A Materials and methods

4A.1 Introduction

To find out if the increase in $\delta^{15}N$ values is restricted to Pleistocene mammoths or if the increased values are characteristic for elephantidae, $\delta^{15}N$ values of modern elephants, rhinoceroses, and horses were studied and evaluated. Finally, the patterns of the $\delta^{15}N$ values of the modern and Pleistocene animals were compared with each other in chapter 5.

4A.2 Sample selection

For this research it was important that certain conditions of the animals under study were known, such as the feeding pattern, the climatological circumstances, and the age. This is the only way in which the $\delta^{15}N$ values could be compared with each other correctly.

The majority of stable nitrogen and carbon isotope values of the Pleistocene mammoth, horse and rhinoceros has been measured on bones. Therefore, the initial goal was to obtain the $\delta^{13}C$ and $\delta^{15}N$ values of the modern elephant, rhinoceros and horse from bone collagen as well. However, despite the abundant collection of bones of deceased animals from Dutch zoos at the veterinary science in Utrecht, bone collagen did not seem to be a potential source for measuring stable nitrogen and carbon isotope values. This was because the veterinary science bones all had been treated with preservatives, and this might cause bias in the stable isotope values[3].

In general, the patterns of stable nitrogen and carbon isotope values which are found in bone collagen are similar to those found in body tissues such as nail, hair and

[3] The potential of bone as a chemical signal for past cultural and ecological relationships should is not yet an integral part of programs for both recovery and curation (Moore *et al*. 1989, 444), for many bones are treated with conservatives that cause bias the isotope values. To find out if treated bones indeed show different isotope values than untreated bones, Van der Plicht (2009) measured two bone samples of a 2 years old cow provided by the Faculty of Veterinary Science of the Utrecht University. In Utrecht, one of the samples remained untreated and the other sample was immersed in water at 80°C during 24 hours, and accordingly bleached in 2% H_2O_2 during one week. The treated bone sample contained about half (20.4) the C% of the fresh bone (45.3). Moreover, the treated bone showed depletion for both $\delta^{13}C$ (±4‰) and $\delta^{15}N$ (±2‰) value relative to the fresh (untreated) bone. These results show that stable isotope values in bone collagen are changed after treatment with aggressive conservatives.

skin (Sponheimer *et al*. 2003a, 84-5). Although there might be enrichment or depletion between the values of nail and bone (O'Connell *et al*. 2001, 1253), patterns will be comparable. While the nails of horses, elephants, and rhinoceroses living in captivity have to be trimmed and balanced regularly, nails emerged to be an efficient sample material for this study.

4A.3 Samples

There were two opportunities to measure samples in the CIO laboratory in Groningen in October and November 2008. In October, samples that were collected in July, August and September were measured. In this period, the mean temperature was 16.37°C and the mean precipitation was 91.33 mm in the Netherlands. Samples collected in October and early November were measured in November. In October and November the mean temperature was 8.50°C, thus almost half the temperature of the period July-September. The mean precipitation was also lower than in the period before: 88.00 mm in the Netherlands. Information about temperature and precipitation was provided by the KNMI.

In the period from July until September samples were derived from the hoofs of ten equids: seven horses (*Equus caballus*), two zebras (*Equus quagga boehmi*), and one przewalski horse (*Equus ferus przewalskii*). Those were derived from several Dutch zoos, riding schools, and a horse-smith. The samples compiled nail and horn material from six rhinoceroses, namely four *Ceratotherium simum* and two *Rhinoceros unicornis*. The thirteen samples from elephants were derived from the nails and soles of the feet from thirteen elephants from different Dutch zoos and comprised ten *Elephas maximus* and three *Loxodonta africana Africana*. An overview of those samples is given in Table 2.

In Table 3, the samples which were collected in October and November are shown. During this period, unfortunately no rhinoceros samples could be gathered. However, samples of hoofs from ten horses (*Equus caballus*) were taken, from privately-owned individuals and from animals from riding schools. Nine elephants (*Elephas maximus*) that had been trimmed in the period from July till September provided samples for the period October/November.

The most important ingredient of the diets of the elephants, rhinoceroses and horses sampled is fresh grass and/or hay. They are also fed some special horse or pachyderm pellets which contain additional nutrients including proteins and vitamins. The elephants and rhinoceroses eat some fruits, vegetables, and sometimes branches. For each individual detailed dietary information and the composition of the horse and pachyderms pellets is provided in Appendix C.

Table 2 Samples collected in the period from July until September 2008. 'x' indicates that sex and/or age was unknown.

Sample name	Source	Species	Sex f/m	Age	Sample number
Chuckie	Riding school, Groningen	*Equus caballus*	x	x	45768
Tanja	Riding school, Slootdorp	*Equus caballus*	x	x	45769
Przwalskipaard	Zoo, Beekse Bergen	*Equus ferus przewalskii*	x	x	45770
Lorette	Riding School, Oegstgeest	*Equus caballus*	f	10/12	45771
Taila	Riding School, Oegstgeest	*Equus caballus*	f	27/28	45772
Domenico	Riding School, Oegstgeest	*Equus caballus*	m	old	45773
Kondor	Riding School, Oegstgeest	*Equus caballus*	m	19	45774
Azira	Zoo, Emmen	*Equus quagga boehmi*	f	1	45775
Sheshe	Zoo, Emmen	*Equus quagga boehmi*	f	1	45776
Neushoorn Beekse Bergen	Zoo, Beekse Bergen	*Ceratotherium simum*	x	x	45777
Zimon	Zoo, Amersfoort	*Rhinoceros unicornis*	m	9	45778
Petra	Zoo, Emmen	*Ceratotherium simum*	f	27	45779
Jennifer	Zoo, Emmen	*Ceratotherium simum*	f	38	45780
Kusini	Zoo, Emmen	*Ceratotherium simum*	m	16	45781
Namaste	Zoo, Blijdorp	*Rhinoceros unicornis*	f	19	45782
Timber	Zoo, Blijdorp	*Elephas maximus*	m	10	45783

Sibu	Zoo, Dierenrijk	*Loxodonta africana africana*	m	4	45784
Kan Kaung	Zoo, Dierenrijk	*Loxodonta africana africana*	m	6	45785
Jula	Zoo, Amersfoort	*Elephas maximus*	f	x	45786
Mimi	Zoo, Amersfoort	*Elephas maximus*	f	x	45787
War War	Zoo, Amersfoort	*Elephas maximus*	f	x	45788
Mingalar Oo	Zoo Emmen	*Elephas maximus*	f	17	45789
Ma Yay Yee	Zoo Emmen	*Elephas maximus*	f	10	45790
Annabel	Zoo Emmen	*Elephas maximus*	f	45	45791
Yuzin	Zoo Emmen	*Elephas maximus*	f	29	45792
Radza	Zoo Emmen	*Elephas maximus*	m	42	45793
Swe San Htay	Zoo Emmen	*Elephas maximus*	f	30	45794
Olifant Beeksebergen	Zoo, Beekse Bergen	*Loxodonta africana africana*	x	x	45795
Paard Wassenaarseweg Katwijk	Horse-smith, Katwijk	*Equus caballus*	x	x	45796

Table 3 Samples collected in October and November 2008. 'x' indicates that sex and/or age is unknown.

Sample name	Source	Species	Sex f/m	Age	Sample number
Mike 1	Private, Mike	*Equus caballus*	f	15	46192
Mike 2	Private, Mike	*Equus caballus*	m	15	46193
Sepp	Riding School, Oegstgeest	*Equus caballus*	m	18	46194
Lobke 1	Riding School, Oegstgeest	*Equus caballus*	x	x	46195
Lobke 2	Riding School, Oegstgeest	*Equus caballus*	x	x	46196
Lobke 3	Riding School, Oegstgeest	*Equus caballus*	x	x	46197
Lobke 4	Riding School, Oegstgeest	*Equus caballus*	x	x	46198
Lobke 5	Riding School, Oegstgeest	*Equus caballus*	x	x	46199
Lobke 6	Riding School, Oegstgeest	*Equus caballus*	x	x	46200
Lobke 7	Riding School, Oegstgeest	*Equus caballus*	x	x	46201
War War	Zoo, Amersfoort	*Elephas maximus*	f	x	46202
Mimi	Zoo, Amersfoort	*Elephas maximus*	f	x	46203
Swe San Htay	Zoo Emmen	*Elephas maximus*	f	30	46204
Mingalar Oo	Zoo Emmen	*Elephas maximus*	f	17	46205

Yuzin	Zoo Emmen	*Elephas maximus*	f	29	46206
Annabel	Zoo Emmen	*Elephas maximus*	f	45	46207
Radza	Zoo Emmen	*Elephas maximus*	m	42	46208
Ma Yay Yee	Zoo Emmen	*Elephas maximus*	f	10	46209
Timber	Zoo, Blijdorp	*Elephas maximus*	m	10	46210

4A.4 Sample preparation

Each nail sample was cut into pieces varying from 0.5 to 4 cm so the material would fit into cups, on which the sample numbers were written. Next, each sample was cleaned in a solution of 10% HCL (4% HCL appeared to be insufficient). After the HCL solution was removed, each sample was thoroughly cleansed with purified water. The samples were sufficiently clean if the water, in which the samples were rinsed, was pH neutral. Then, the water was strained off and the samples were placed in a stove at a temperature of 100°C during the next twelve hours, for the samples needed to be absolutely dry. The procedure mentioned above is the same as the one used for collagen preparation for 14C dating.

After the samples were taken out of the stove and cooled down, they were cut into minuscule fragments. To measure the $\delta^{13}C$ value, 5mg material had to be extracted from each sample and wrapped up in a small piece of tin foil. The $\delta^{15}N$ values needed to be measured in duplicate, and for each measurement 1.20mg of sample material was required. By means of a digital analytical balance the mass of each sample could be determined precisely.

The tin cups containing the collagen were placed in an Elemental Analyser (EA). First they were combusted at a temperature of 1000 °C in an automatically combustion system. Then, they entered a gas chromatographic column (GC column), which separated the gasses. After a run of CO_2 for $\delta^{13}C$ through the GC column, the mass spectrometer (which is connected to the elemental analyser) measured the isotope ratios. Finally, another run through the GC column was realized by the analyst; this time concerning N_2 for $\delta^{15}N$. In the B-part of this chapter, the results of the measurements are presented; these will be discussed further in chapter 5.

4A.5 Analysing the results of the samples

Besides the $\delta^{15}N$ and $\delta^{13}C$ values, the elemental analyser provides C% and N%. To determine the reliability of the values the C/N ratio ($\frac{C\%}{N\%} * \frac{14}{12}$) was estimated. Good quality bone collagen provides C/N ratios of about 3.

Afterwards, to visualize the values, they were plotted on scatterdiagrams. It seemed that some of the values differed remarkably from the other values. To see if these values were acceptable or 'outliers', potential outliers in both $\delta^{13}C$ and $\delta^{15}N$ values were traced using a boxplot.

At first sight, the values on the scatterdiagrams for the different animal groups (elephant, rhinoceros, and horse) seemed to be more or less all clustered together. However, the scatterdiagrams showed difference in the abundance of the spreads, and the rhinoceroses seemed to have $\delta^{15}N$ values a little lower than the elephants and horses. By means of the Mann-Whitney U test the significance of the difference between the different animal groups was calculated.

The statistical methods and the calculations and their results are discussed in detail in Appendix D.

Chapter 4B Results

4B.1 Measurements

All samples measured provided both a $\delta^{13}C$ and $\delta^{15}N$ value, as mentioned above (). One sample (horse, Domenico Lobke) did not provide a $\delta^{13}C$ value in first instance, but a second measurement on this sample determined the $\delta^{13}C$ value being -25.27‰.

To be reliable, a nail measurement should have a C/N ratio of about 3.4 (O'Connell *et al*. 1999, 415; O'Connell *et al*. 2001, 1250). Though the mean C/N ratios for rhinoceros and horse are 3.37 and 3.53 respectively, many elephants have remarkably high C/N ratios (between 3.29 and 5.76) with a mean C/N ratio of 4.27. Samples with C/N ratios higher than 4.00 are mentioned in italic.

Table 4 Overview of all samples. In the fifth column the C/N ratio is shown. 'xx' concerns the averaged values of elephants; here a C/N ratio is irrelevant. High C/N ratios (>4.00) are written in italic. Em=*Elephas maximus*, Laa=*Loxodonta africana Africana*, Ec=*Equus caballus*, Efp=*Equus ferus przewalskii*, Eqb=*Equus quagga boehmi*, Cs=*Ceratotherium simum*, Ru= *Rhinoceros unicornis*

Sample name	$\delta^{13}C$	$\delta^{15}N$	C/N ratio	Animal	Species
Timber Blijdorp	*-26.92*	*5.30*	*4.50*	*Elephant*	*Em*
Jula Amersfoort	-26.85	7.94	3.97	Elephant	Em
Mimi Amersfoort	-26.44	8.04	3.82	Elephant	Em
War War Amersfoort	-26.07	7.95	3.55	Elephant	Em
Mingalar Oo Emmen	-25.96	6.65	3.29	Elephant	Em
Ma Yay Yee Emmen	*-26.48*	*6.40*	*4.54*	*Elephant*	*Em*
Annabel Emmen	*-26.14*	*6.89*	*5.76*	*Elephant*	*Em*
Yuzin Emmen	*-26.20*	*6.61*	*4.14*	*Elephant*	*Em*
Radza Emmen	*-26.88*	*6.66*	*4.83*	*Elephant*	*Em*
Swe San Htay Emmen	*-27.03*	*6.52*	*4.55*	*Elephant*	*Em*
War War Amersfoort	*-26.65*	*7.67*	*4.15*	*Elephant*	*Em*
Mimi Amersfoort	*-26.20*	*7.80*	*4.50*	*Elephant*	*Em*
Swe San Htay Emmen	*-27.11*	*6.15*	*4.49*	*Elephant*	*Em*
Mingalar Oo Emmen	*-26.29*	*6.19*	*4.01*	*Elephant*	*Em*
Yuzin Emmen	-25.66	6.54	3.48	Elephant	Em
Annabel Emmen	-25.89	7.01	3.85	Elephant	Em
Radza Emmen	*-26.17*	*6.36*	*4.38*	*Elephant*	*Em*
Ma Yay Yee Emmen	*-26.35*	*6.71*	*4.49*	*Elephant*	*Em*
Timber Blijdorp	*-27.22*	*5.96*	*4.49*	*Elephant*	*Em*
War War average	-26.36	7.81	xx	Elephant	Em
Mimi average	-26.32	7.92	xx	Elephant	Em
Swe San Htay average	-27.07	6.34	xx	Elephant	Em
Mingalar Oo average	-26.13	6.42	xx	Elephant	Em

Yuzin average	-25.93	6.58	xx	Elephant	Em
Annabel average	-26.02	6.95	xx	Elephant	Em
Radza average	-26.53	6.51	xx	Elephant	Em
Ma Yay Yee average	-26.42	6.55	xx	Elephant	Em
Timber average	-27.07	5.63	xx	Elephant	Em
Sibu Dierenrijk	*-26.91*	*5.81*	*4.20*	*Elephant*	*Laa*
Kan Kaung Dierenrijk	*-26.02*	*5.92*	*4.26*	*Elephant*	*Laa*
Elephant Beeksebergen	*-25.75*	*5.98*	*4.70*	*Elephant*	*Laa*
Chuckie Groningen	-25.86	7.42	3.44	Horse	Ec
Tanja Slootdorp	-25.49	7.61	3.36	Horse	Ec
Lorette Lobke	-25.57	7.30	3.86	Horse	Ec
Taila Lobke	-26.41	7.75	3.37	Horse	Ec
Domenico Lobke	-25.27	7.56	3.66	Horse	Ec
Kondor Lobke	-26.01	7.47	3.56	Horse	Ec
Wassenaarseweg Katwijk	-26.88	8.09	3.34	Horse	Ec
Mike 1	-24.96	6.90	3.43	Horse	Ec
Mike 2	-23.68	7.75	3.50	Horse	Ec
Lobke Sepp	*-25.96*	*7.53*	*4.07*	*Horse*	*Ec*
Lobke 1	-25.15	8.24	3.36	Horse	Ec
Lobke 2	-25.91	6.81	3.27	Horse	Ec
Lobke 3	-26.17	9.81	3.44	Horse	Ec
Lobke 4	-26.58	7.63	3.60	Horse	Ec
Lobke 5	-25.82	8.39	3.63	Horse	Ec
Lobke 6	-26.68	7.48	3.61	Horse	Ec
Lobke 7	-26.06	7.30	3.62	Horse	Ec
Przwalski Beeksebergen	-26.05	7.77	3.29	Horse	Efp
Aziza Emmen	-26.27	5.83	3.73	Horse	Eqb
Sheshe Emmen	-26.16	5.61	3.38	Horse	Eqb
Rhinoceros Beeksebergen	-23.00	6.30	3.42	Rhinoceros	Cs
Petra Emmen	-26.04	6.33	3.19	Rhinoceros	Cs
Jennifer Emmen	-26.82	5.93	3.37	Rhinoceros	Cs
Kusini Emmen	-25.54	6.57	3.60	Rhinoceros	Cs
Zimon Amersfoort	-25.22	7.12	3.22	Rhinoceros	Ru
Namaste Blijdorp	-25.47	5.56	3.43	Rhinoceros	Ru

4B.2 Values

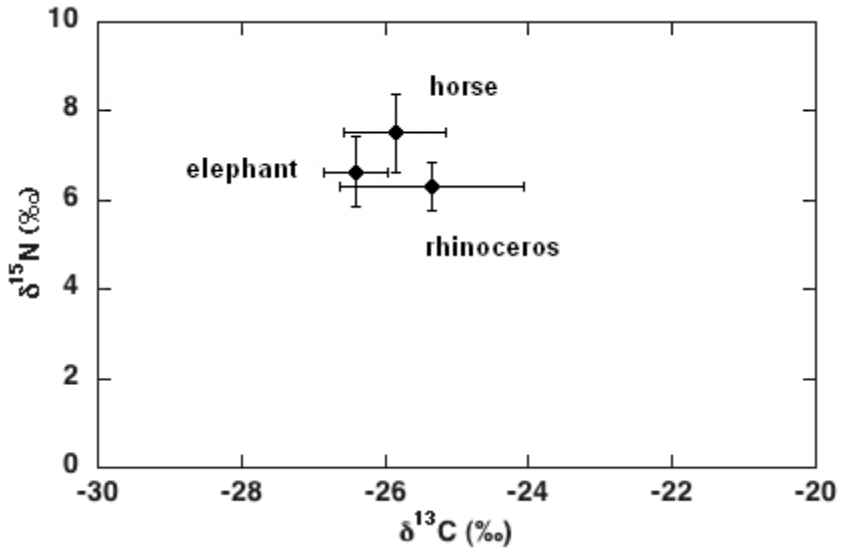

Figure 12 Range of the $\delta^{13}C$ and $\delta^{15}N$ values

Figure 12 shows that the mean $\delta^{13}C$ values of the elephants (-26.41‰), horses (-25.85‰) and rhinoceroses (-25.35‰) are quite similar. However, the extent of the spread of $\delta^{13}C$ values of each animal group does differ: the elephants have a range of 1.32‰, while the rhinoceroses and the horses have a range of 3.82‰ and 3.20‰ respectively.

The mean $\delta^{15}N$ values have also a small spread: elephants 6.64‰, horses 7.51‰, and rhinoceroses 6.30‰. As for $\delta^{13}C$ values, the ranges of the $\delta^{15}N$ values for the different animal groups vary considerably. The horses cover an area of 4.20‰, the elephants 2.31‰, and the rhinoceroses just 1.55‰.

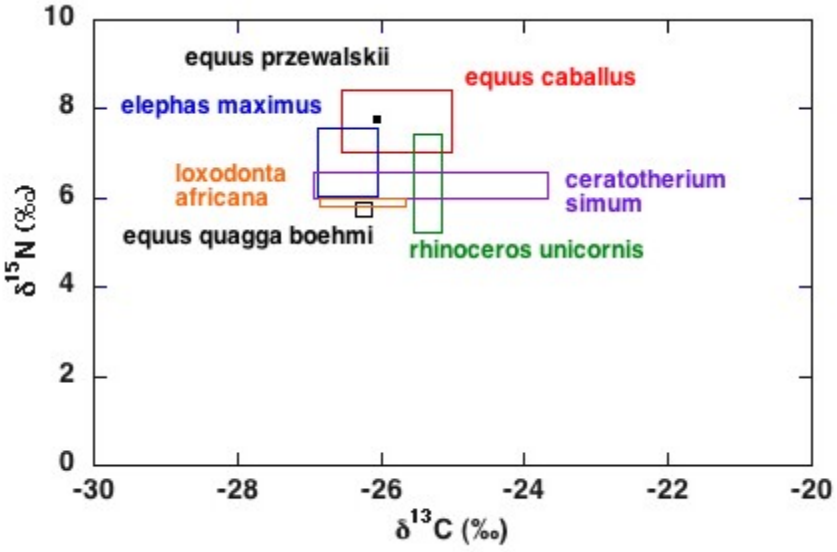

Figure 13 Range of the $\delta^{13}C$ and $\delta^{15}N$ values for the several species

As shown in Figure 13, within the animal groups, hardly any difference exists in the $\delta^{13}C$ values between several species. The *Elephas maximus* and *Loxodonta africana Africana* have averages of 26.47‰ and -26.23‰. Also, the average $\delta^{13}C$ values of *Equus caballus* (-5.79‰), *Equus ferus przewalskii* (-26.05‰), and *Equus quagga boehmi* (-26.22‰) are quite similar. Both *Ceratotherium simum* and *Rhinoceros unicornis* have an average $\delta^{13}C$ value of -25.35‰.

Although the mean $\delta^{15}N$ values of *Ceratotherium simum* (6.28‰) and *Rhinoceros unicornis* (6.33‰) show hardly any difference, within the elephants and horses significant variance exists between the different species. The $\delta^{15}N$ values of *Elephas maximus* are somewhat higher than those of *Loxodonta africana Africana*, with mean values of 6.82‰ and 5.90‰ respectively. Amongst the horses, most samples were derived from *Equus caballus*, having a mean $\delta^{15}N$ value of 7.71‰, which is quite similar to the value of the *Equus ferus przewalskii* (7.77‰). However, the mean $\delta^{15}N$ value of *Equus quagga boehmi* lies about 2‰ lower.

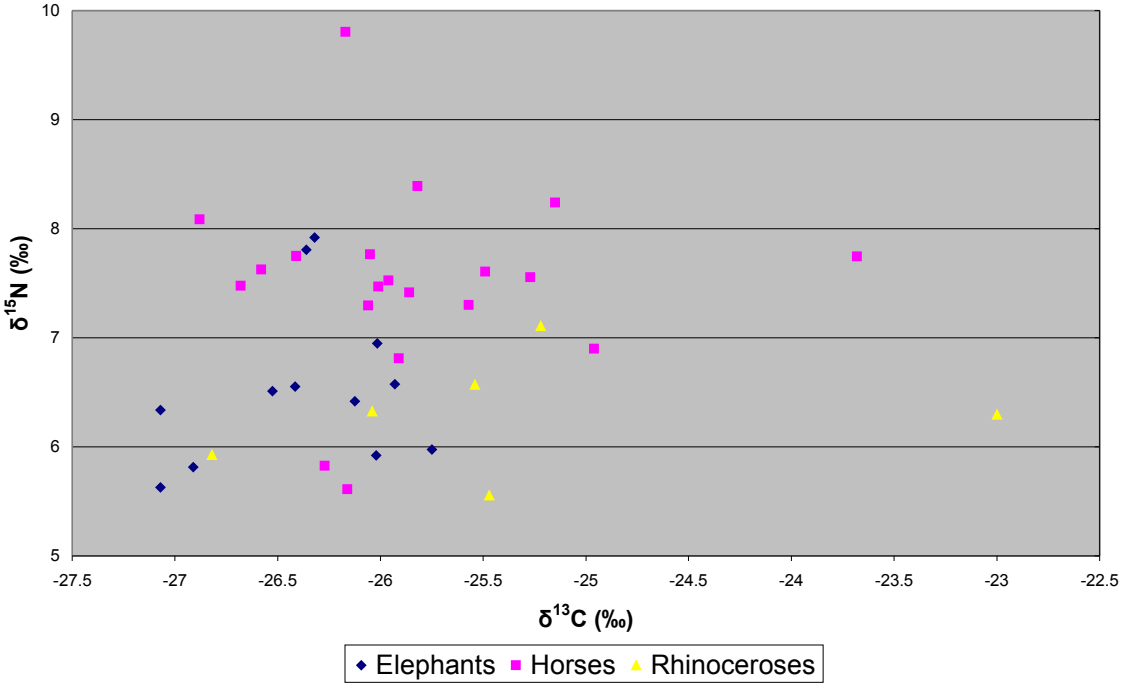

Figure 14 Overview of the values derived from horses, elephants and the rhinoceroses in the period July-October. Values of multiple samples of the same individual have been averaged.

4B.3 Outliers

In Figure 14, the values of all samples shown in were plotted. The $\delta^{13}C$ and $\delta^{15}N$ values are indicated on the X-axis and the Y-axis respectively. The scatterdiagram (Figure 14) shows a cluster of values at the left bottom corner. One horse has an elevated $\delta^{15}N$ value (9.81‰) compared with the others. Moreover, two $\delta^{13}C$ values are relatively higher than the rest: a horse and a rhinoceros having $\delta^{13}C$ values of -23.68‰ and -23.00‰ respectively. These are determined as being 'outliers' (see Appendix D) and will be excluded from further study in this thesis. The two horses are outliers for unknown reason. The nature of this rhinoceros sample is questionable, which might be the explanation for being an outlier.

4B.4 July-September vs. October-November

Table 5 Values of samples taken in the first period (July, August, and September 2008)

First samples (Jul/Aug/Sep)	n	average		standard deviation		(min;max)	
		$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
elephants	**13**	**-26.43**	**6.67**	**0.44**	**0.74**	**(-27.03;-25.96)**	**(5.30;8.04)**
Elephas maximus	10	-26.50	6.89	0.40	0.86	(-27.03;-25.96)	(5.30;8.04)
Loxodonta africana africana	3	-26.23	5.90	0.61	0.08	(-26.91;-25.75)	(5.815;5.92)
horses	**10**	**-26.00**	**7.24**	**0.48**	**0.83**	**(-26.88;-25.27)**	**(5.61;8.00)**
Equus caballus	7	-25.93	7.60	0.56	0.26	(-26.88;-25.27)	(7.30;8.09)
Equus ferus przewalskii	1	-26.05	7.77	x	x	x	x
Equus quagga boehmi	2	-26.22	5.72	0.08	0.15	(-26.27;-26.16)	(5.61;5.83)

Table 6 Values of samples taken in the second period (October and November 2008)

Second samples (Oct/Nov)	n	average		Standard deviation		(min;max)	
		$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
elephants	**10**	**-26.39**	**6.71**	**0.52**	**0.66**	**(-27.22;-25.66)**	**(5.96;7.80)**
Elephas maximus	9	-26.39	6.71	0.52	0.66	(-27.22;-25.66)	(5.96;7.80)
horses	**8**	**-25.89**	**7.53**	**0.60**	**0.56**	**(-26.68;-24.96)**	**(6.81;8.39)**
Equus caballus	8	-25.89	7.53	0.60	0.56	(-26.68;-24.96)	(6.81;8.39)

Table 5 and Table 6 show the number of samples taken, and the average values, standard deviations, and range for the first and second sample periods respectively. One can see that there is no significant difference between the mean $\delta^{13}C$ values of horses and elephants in the different periods. The mean $\delta^{15}N$ values of elephants and horses do not show much difference either. Despite the small difference in mean values between the two periods regarding elephants and horses, depletion is visible in the mean $\delta^{13}C$ values in the second period relative to the first, while the mean $\delta^{15}N$ values show enrichment.

Figure 15 and Figure 16 show the $\delta^{15}N$ values and $\delta^{13}C$ values of *Elaphus maximus* derived from the first and second period are indicated for comparison respectively. Figure 15 and Figure 16 show that no general shift (albeit enrichment or depletion) is visible in either $\delta^{15}N$ values or $\delta^{13}C$ values between the first and second period. In the second period, four individuals show enrichment in $\delta^{15}N$ values relative to the first period (Timber, Ma Yay Yee, Annabel, and Swe San Htay), whereas the other five elephants show depletion. Figure 16 shows that four elephants have enriched $\delta^{13}C$

values relative to the first period (Timber, War War, Mingalar Oo, and Swe San Htay). The other five elephants show depleted $\delta^{13}C$ values relative to the first period.

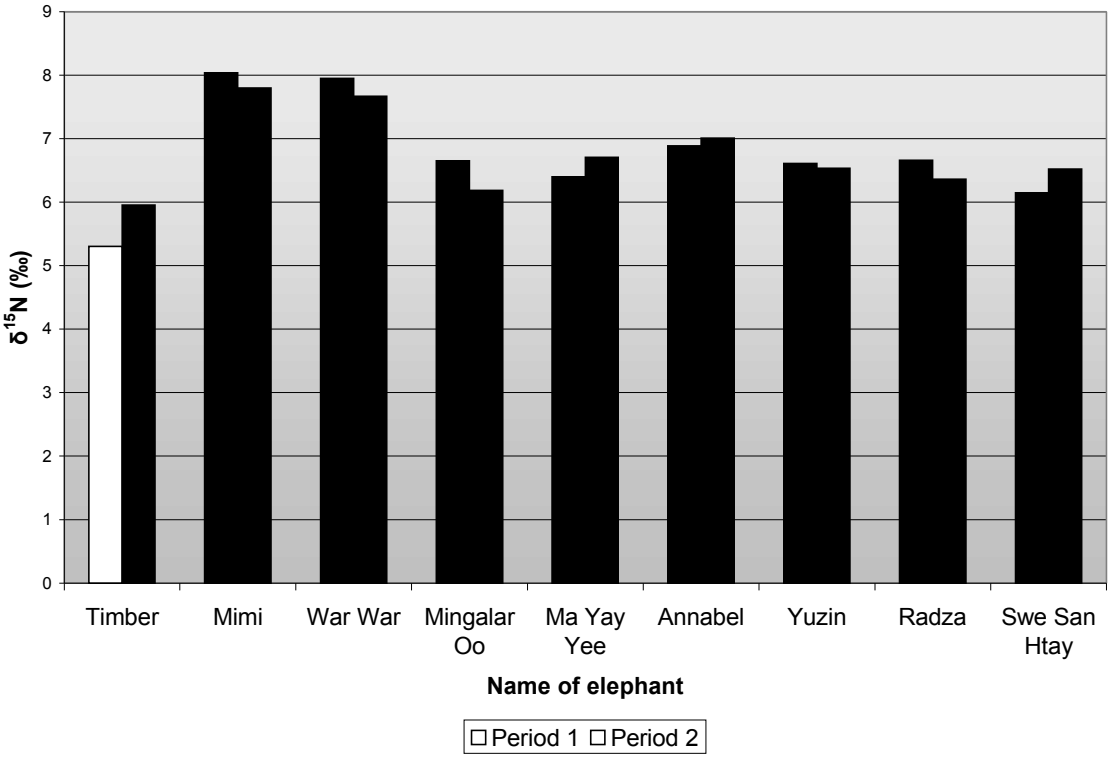

Figure 15 Comparison between period 1 (July, August, and September 2008) and 2 (October and November 2008) of $\delta^{15}N$ values of *Elaphus maximus*.

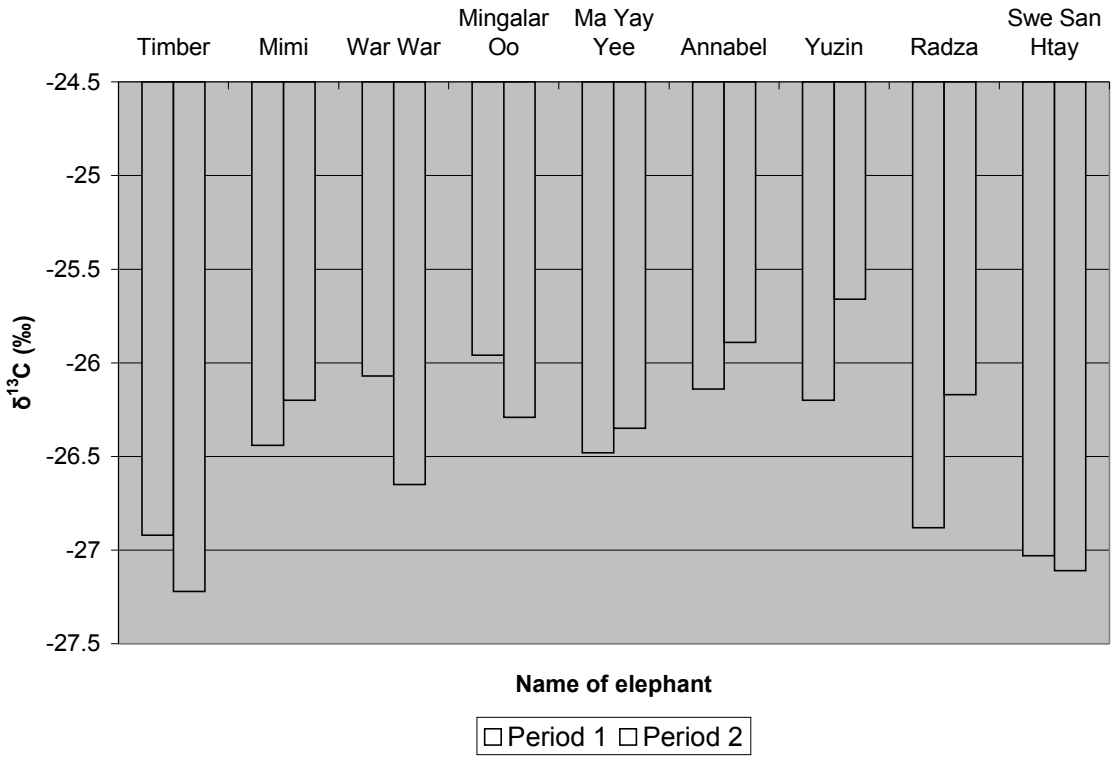

Figure 16 Comparison between period 1 (July, August, and September 2008) and 2 (October and November 2008) of $\delta^{13}C$ values of *Elaphus maximus*.

4B.5 Significance of difference between animal groups

As mentioned previously, both $\delta^{15}N$ values and $\delta^{13}C$ values seem at first to be quite similar. The Mann-Whitney U test was used to estimate whether the distribution of $\delta^{15}N$ and $\delta^{13}C$ values of the different animal groups was indeed equal, or whether they differed significantly.

The principle of the Mann-Whitney U test and the calculations on each animal group is stated in Appendix D. According to the Mann-Whitney U test, both stable nitrogen and carbon values of the horses differ significantly to those of the elephants. Also, the horses differ significantly relative to the rhinoceroses in both their $\delta^{15}N$ and $\delta^{13}C$ values. Between the elephant group and the rhinoceroses there was not a significant difference either for the $\delta^{15}N$ or $\delta^{13}C$ values.

4B.6 Comparing values of different zoos

Figure 17 shows the $\delta^{13}C$ and $\delta^{15}N$ values for elephants in each zoo. The $\delta^{13}C$ ranges do not show considerable variation between the elephants of the different zoos. In contrast, amongst the $\delta^{15}N$ values different clusters are visible for each zoo: the elephants from Amersfoort have the highest values (7.81-7.94‰) and the elephant from Blijdorp has the lowest $\delta^{15}N$ value (5.63‰). In between these are the elephants from Dierenrijk (5.82-5.92‰), Beeksebergen (5.98‰), and Emmen (6.34-6.95‰). Per zoo, the $\delta^{15}N$ value ranges are considerably small.

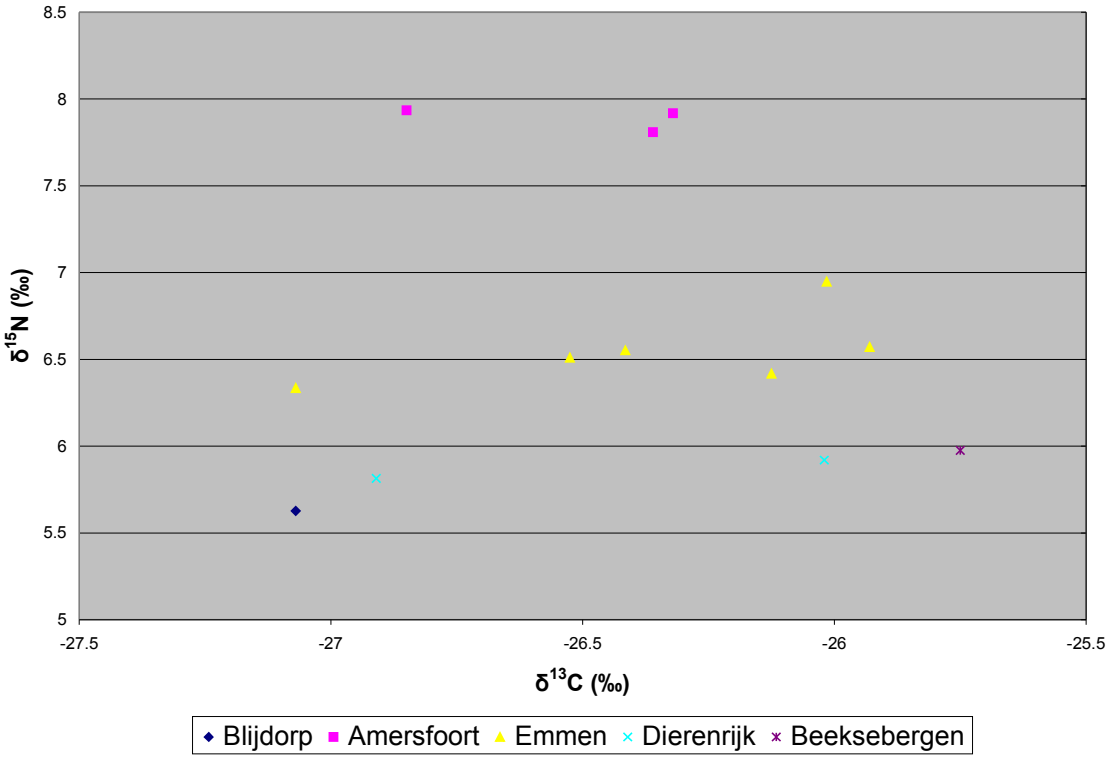

Figure 17 $\delta^{13}C$ and $\delta^{15}N$ values of elephants in each zoo

Figure 18 shows the $\delta^{13}C$ and $\delta^{15}N$ values for rhinoceroses in each zoo. Although the number of rhinoceros samples is low, a pattern comparable to that of the elephants is visible. Similar to the elephants, their $\delta^{13}C$ ranges are considerably small and do not show any special distinction between the different zoos. Moreover, the rhinoceros from Amersfoort has the highest $\delta^{15}N$ value (7.11‰) and the rhinoceros from Blijdorp has the

lowest value (5.55‰). The rhinoceroses from Emmen are situated in between with $\delta^{15}N$ values ranging from 5.93‰ to 6.57‰.

Both the $\delta^{13}C$ and $\delta^{15}N$ values of horses are clustered together and do not show any special characteristics for each zoo or stable: see Figure 19. The only exceptions seem to be the two zebras from Emmen Zoo, with significantly lower values (5.61‰ and 5.83‰).

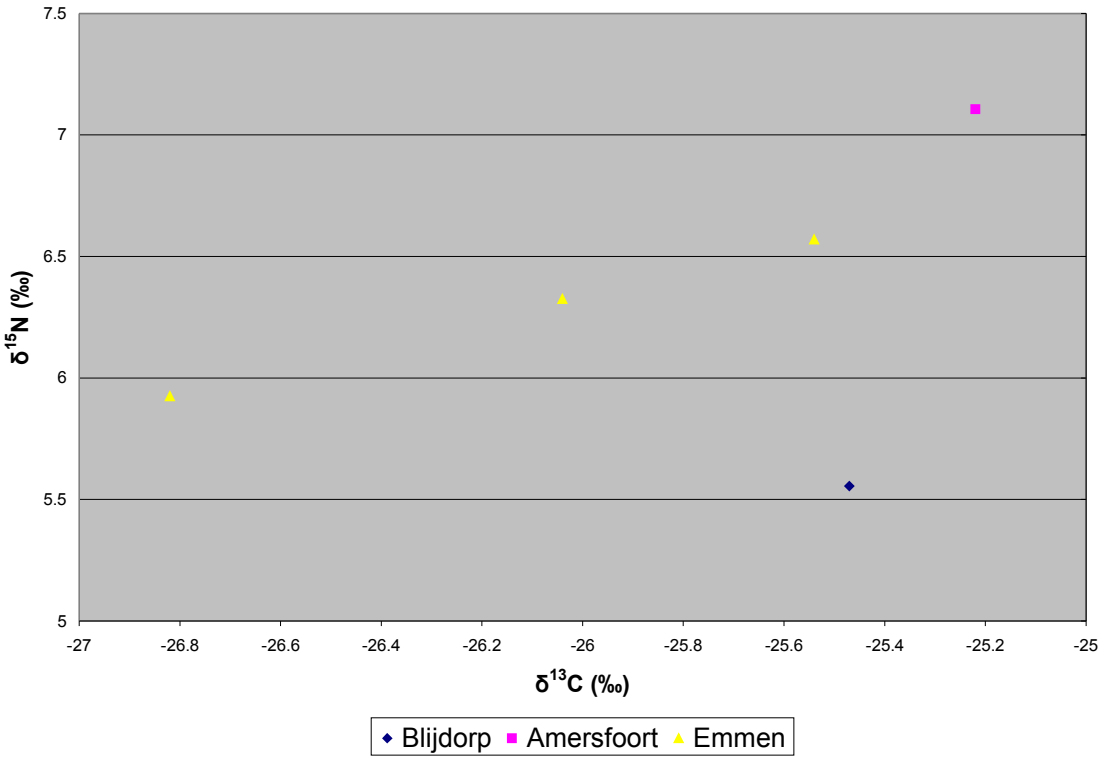

Figure 18 $\delta^{13}C$ and $\delta^{15}N$ values of rhinoceroses in each zoo

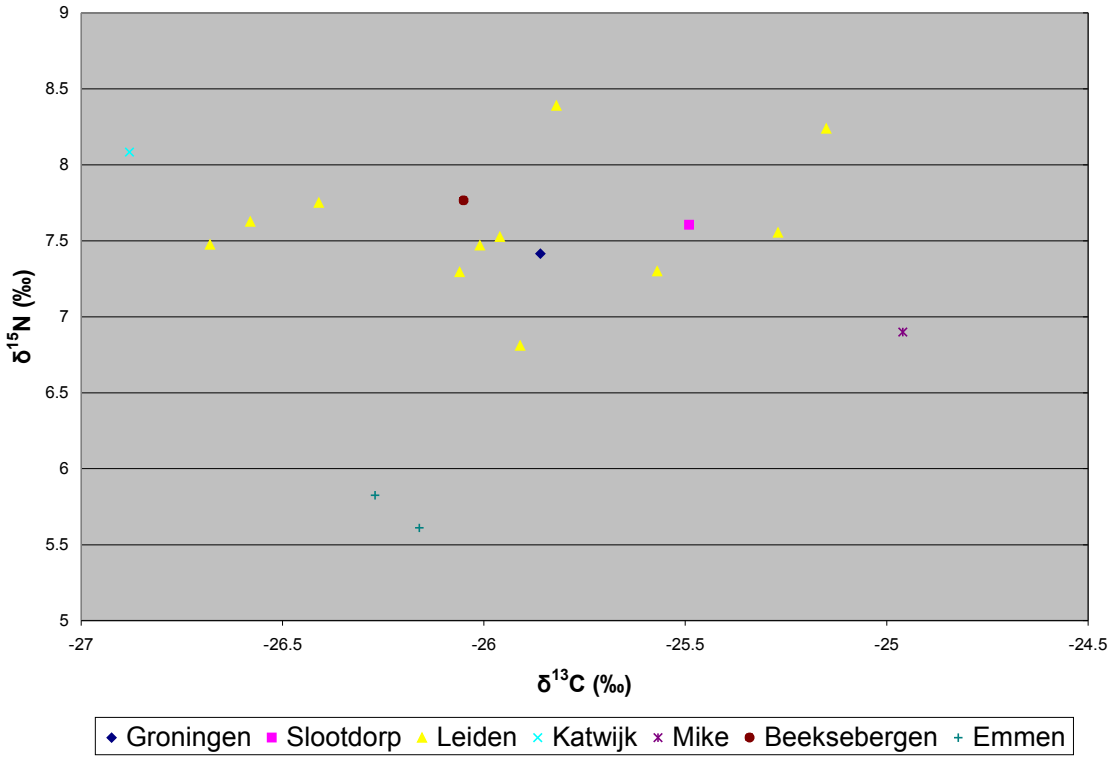

Figure 19 $\delta^{13}C$ and $\delta^{15}N$ values of horses in each zoo/stable

4B.7 Weather conditions, age, sex and reproduction

During the total period (July-October), both temperature and precipitation have not been significantly high or low that it might have caused stress (e.g. heat, cold, or water stress) amongst the animals.

Although the temperature in the period July-September is almost double the temperature in the period October-November, the values do not show any clear depletion or enrichment in the second period relative to the first.

To be able to exclude the factor of suckling, none of the present samples are derived from suckling individuals, or animals that had been suckling during the six months previously to the sampling period.

The rhinoceros Namaste of Blijdorp had calved just the night before sampling, so she had been pregnant during some months previously to the sampling period.

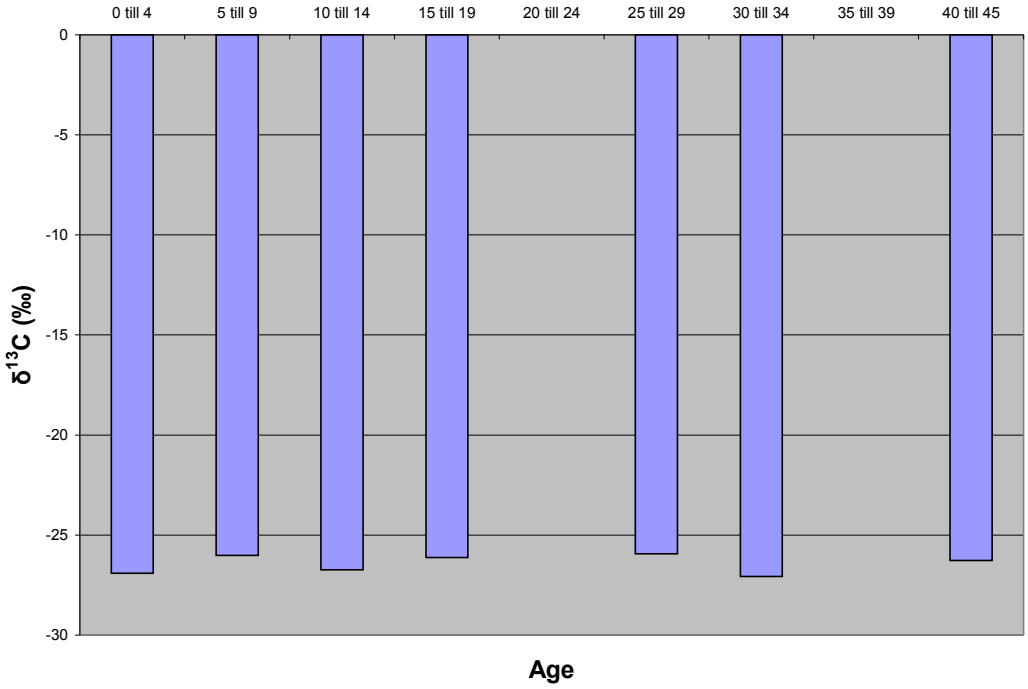

Figure 20 $\delta^{13}C$ values in relation to age of the elephants

As is visible in Figure 20 and Figure 21 respectively, no clear relationship exists between the $\delta^{13}C$ and $\delta^{15}N$ values and the age of the elephants.

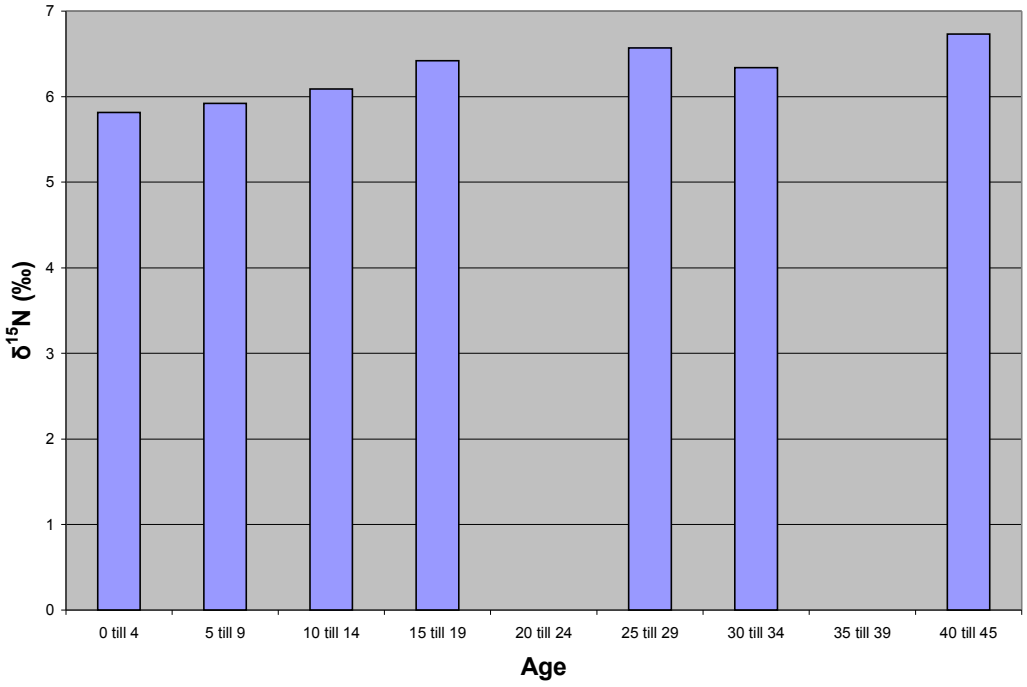

Figure 21 $\delta^{15}N$ values in relation to age of the elephants

Figure 22 does not show a clear relationship between sex and the stable isotope ratios of the herbivores. The $\delta^{15}N$ values of elephant females seem higher than these of elephant males. However, the $\delta^{15}N$ values of horse males and rhinoceros males are slightly enriched compared to females. No difference is visible in $\delta^{13}C$ values between males and females for elephants, horses, and rhinoceroses.

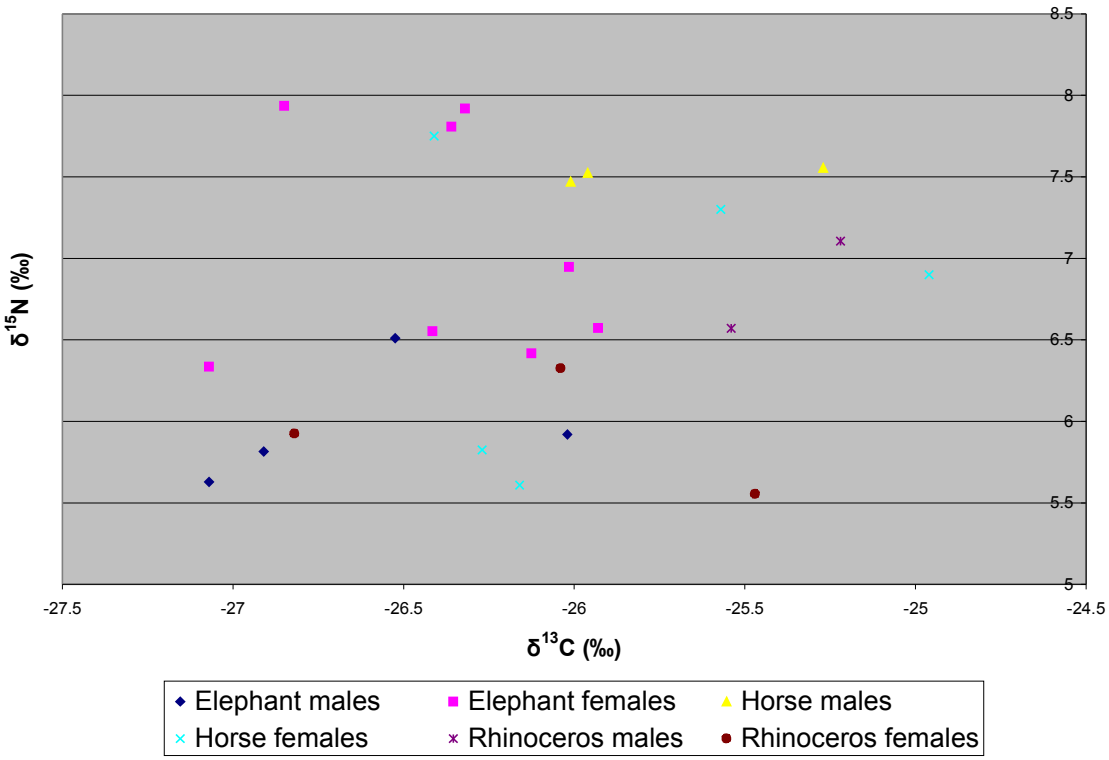

Figure 22 $\delta^{15}N$ and $\delta^{13}C$ values to elephants, horses, and rhinoceroses in relation to sex

Chapter 5 Discussion and conclusions

In Chapter 2, two potential distribution patterns (A and B) of the $\delta^{13}C$ and $\delta^{15}N$ were indicated. One of the hypothetical models (see figure 4B) is that the $\delta^{15}N$ values for elephants are significantly higher than horse and rhinoceros. If this model were right, the modern elephant could be used as an analogue for the Pleistocene mammoth, since the $\delta^{15}N$ and $\delta^{13}C$ values of the Pleistocene mammoth are distributed equally relative to other herbivores. The other model (A) shows a situation where the values of modern horse, rhinoceros, and elephant overlap. If this were the case, the elephant, in a controlled feeding study, cannot be considered as a reliable analogue for the Pleistocene mammoth.

The latter model (A) does approach the results of the research more than the previous: the values of the elephants are not extremely high compared to the values of horse and rhinoceros and hence, do not correspond with model B. The $\delta^{15}N$ values of the three animal groups show considerable overlap. However, albeit small, significant difference exists between both the $\delta^{15}N$ and $\delta^{13}C$ values of the horses and these of the other two animals. The range of both the $\delta^{15}N$ and $\delta^{13}C$ values of the horses is larger than these of the other data. The horses have on the average higher $\delta^{15}N$ values: 78% of these are higher than 7‰. Just 20% of the rhinoceroses have $\delta^{15}N$ values higher than 7‰ and in elephants it is even less (17%). No significant difference exists between the $\delta^{15}N$ and $\delta^{13}C$ values of elephants and rhinoceroses. Moreover, the $\delta^{13}C$ values of the horses are more equally distributed across the total range of these values than those of the elephants and rhinoceroses.

The results obtained suggest that modern elephants differ from Pleistocene mammoth, since the stable isotope distribution patterns of Pleistocene mammoths and modern elephants do not correspond. However, this conclusion might be too simplistic. Life in captivity with controlled and sufficient food supply might have an important impact on the stable isotope values; this may explain the observed differences. To judge this, a number of aspects must be taken into account.

There are a number of similarities between elephants, rhinoceroses and horses. Water uptake and excretion patterns in the modern horse, rhinoceros and elephant are

similar to each other (Clauss *et al*. 2005, 12). The animals are all obligate drinkers, which means that they are not drought tolerant, in contrast to water conserving species (Ambrose 1991, 311; Ambrose *et al*. 1986, 403; Sealy *et al*. 1987, 2711). Furthermore, the digestive system of horses, rhinoceroses and elephants is quite similar: they are all hindgut fermenters using cecal digestion (Wittemyer *et al*. 2008, 8; Sealy *et al*. 1987, 2713; Sponheimer *et al*. 2003a, 82). The microbial activity in the digestive tract takes place particularly in the rumen, but also in the large hindgut, and in the small intestine of elephants (Sealy *et al*. 1987, 2713; Clauss *et al*. 2007, 71).

However, there are some physiological dissimilarities amongst these animals. Elephants in particular differ in some aspects. To begin with, the digestive tract of elephants is significantly shorter compared to other herbivores (Clauss *et al*. 2007, 68). This causes considerably lower digestion efficiency for the elephant, when compared to the horse and rhinoceros. Due to their relative short digestive tract, for elephants the time during which the food consumed is exposed to digestion (digesta retention time) is short. In general, the digestive tract, and, therefore, digesta retention times increase with increasing body size: an amount of food needs more time to pass through a longer digestive tract (Olivier 1982, 292-3; Clauss *et al*. 2005, 180). Since rhinoceroses have a much longer ingesta retention time than horses, they can achieve similar digestion coefficients as horses. In contrast, although the elephant's body is much bigger than that of a horse, the digesta retention time of elephants is similar to that of horses. Therefore, elephants have lower digestion coefficients than horses and rhinoceroses: the percentage of ingested food that is excreted in feces is high relative to the part of food that is digested and absorbed (Clauss *et al*. 2005, 180, 229-235; Martin 1982, 262).

But what about differences in food? The diets of Pleistocene animals consisted predominately of grass, which is (quite) protein poor (Clauss *et al*. 2005, 229-30; Olivier 1982, 295). In winter, on the other hand, browsing might have played a far more important role (Putshkov 2003, 369) because during the dry season mature and dry grasses have lower nitrogen contents than dicot leaves (Ambrose *et al*. 1986, 403). The main constituent of the investigated modern animal's diets is grass and hay. In addition, these animals are fed with special pellets containing extra nutrients, among which (crude)

proteins. The biggest difference between the investigated animals and those living in a wild environment is, however, the constant supply of water and food (including important nutrients). In contrast to wild animals, those living in zoos and stables never had any water or food stress. This difference might explain why the $\delta^{15}N$ value patterns of the animals used in the present study do not correspond with those living in the Pleeistocene.

In their natural habitat animals must deal constantly with water and food stress. During such periods of food shortage, there is a negative nitrogen balance: the amount of nitrogen excreted from the body is higher than the amount of nitrogen ingested. Preferential excretion of ^{14}N may result in increased $\delta^{15}N$ values in the body (Adams *et al*. 2000, 605). If lower digestion coefficients result in more stress during periods of food and/or water shortage, elephants might have even more stress in such periods compared to other herbivores. In that case the nitrogen balance would be more negative than in other herbivores and hence, the $\delta^{15}N$ values of elephants are higher.

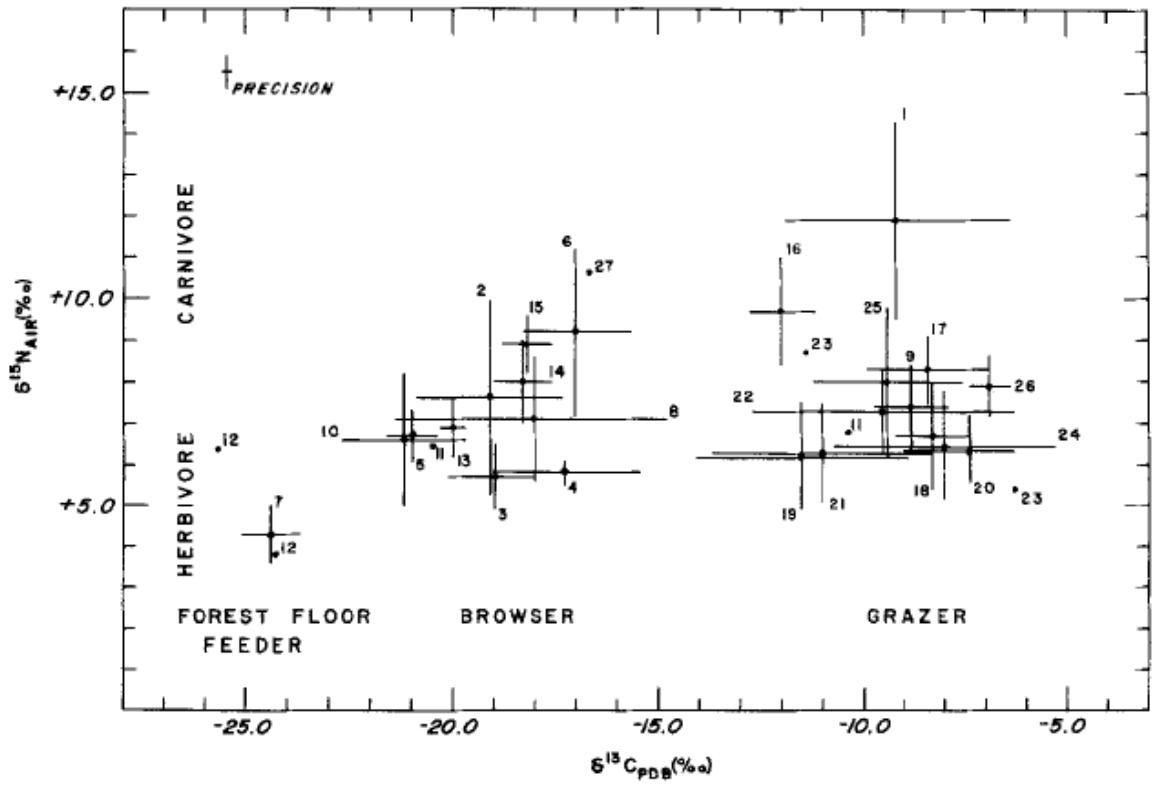

Figure 23 Means and standard deviations of skeletal collagen $\delta^{15}N$ and $\delta^{13}C$ values of East African mammals provided by Ambrose and colleagues in 1986. This diagram definitely shows that the $\delta^{15}N$ value of the elephant (10.6‰) is almost as high as the d15N value of the carnivorous species and is enriched relative to other herbivores. No rhinoceroses' values are available, but zebras are

presented, having an average $\delta^{15}N$ value of 6.7‰. 1= carnivores, 27= elephant (Loxodonta Africana), 18= burchell's zebra. (From: Ambrose *et al*. 1986, 399)

Ambrose *et al*. (1986) showed that in Africa modern animals living in the wild who are obligate drinkers have lower mean $\delta^{15}N$ values than drought-tolerant species, except for elephants and hippopotamuses (Ambrose *et al*. 1986, 399, 403). Figure 23 shows that the $\delta^{15}N$ value of the elephant (10.6‰) is enriched relative to other herbivores, and almost as high as the $\delta^{15}N$ value of the carnivorous species. This pattern shows similarities to that observed in Pleistocene herbivores including mammoths.

Since elephants achieve lower digestion coefficients than most other herbivores, they might experience more stress during periods of water and food shortage compared to these other herbivores. In these circumstances, their nitrogen balance would be more negative than for horses and rhinoceroses, which might be an explanation for the enriched $\delta^{15}N$ values of wild elephants.

The above indicates that the preliminary conclusion, based on the results of the present study, that modern elephants differ from Pleistocene mammoth, might indeed be too simplistic. Taking important aspects as, for instance, food and water stress into account in controlled-feeding studies, it can be concluded that, modern elephants *can* be used to get insight in the mechanisms that determine the observed isotope values in fossil mammoths.

Results obtained in the present study suggest that water and food stress are important factors explaining the significantly higher $\delta^{15}N$ values in elephants and probably Pleistocene mammoths relative to other herbivores. To find out to what extent $\delta^{15}N$ values of elephants are influenced by water and food stress, one would need to measure the $\delta^{15}N$ values in tissues with a short turnover time (e.g. nail or hair) of modern elephants, who endure situations of water and food sufficiency alternated with situations of scarcity. Such measurements are possible via controlled-feeding, if food and water stress can be taken into account. Alternatively, the $\delta^{15}N$ values of wild elephant tissues could be measured at regular intervals. In this way, one is able to estimate and compare the elephant $\delta^{15}N$ values for periods of water and food stress (e.g. the end of the dry season) with values for periods without such stress (e.g. end of the wet season).

An element not mentioned in this thesis so far is a physiological aspect of mammoths. Recent investigation shows (Tikhonov *et al*. in press) that mammoths had a large fat lump in their neck. This might be a physiological adaptation to reduce water and food stress. Maybe, the mammoths were more drought-tolerant than thought. Elephants do not have a comparable body part to store fat, so the influence of such a fat lump on $\delta^{15}N$ values cannot be investigated in elephants. On the other hand, wild camels might be useful in such a study.

In summary, it can be concluded that:

1. The stable isotope value distribution patterns of Pleistocene mammoths and modern elephants from Dutch zoos do not correspond with each other. The $\delta^{15}N$ values of the latter are not enriched relative to coeval living rhinoceroses and horses.
2. Although elephants that live in captivity do not show excessive enrichment in their $\delta^{15}N$ values compared to rhinoceroses and horses, wild modern elephants *show* higher $\delta^{15}N$ values than most other herbivores.
3. It would be possible to carry out controlled-feeding studies with modern elephants and synthesis food and water stress to gain insight into the mechanisms that determine the isotope values observed in fossil mammoths. However, there would be serious ethical considerations for such research.
4. Elephant samples that are derived from the same zoo, show comparable $\delta^{15}N$ values that differ from that of other zoos. Hence, each zoo presents a specific, isolated cluster of elephant $\delta^{15}N$ values. A similar situation is visible for the $\delta^{15}N$ values of rhinoceroses.
5. Age, sex and weather conditions do not affect the stable isotope ratios in modern captive horse, rhinoceros and elephant.
6. Nails of elephants have extremely high C/N ratios (between 3.29 and 5.76 and with a mean C/N ratio of 4.27) in comparison with nails of other herbivores.

The present research raised a number of questions:

- *What is the reason for the extremely high C/N ratios in elephant nails?*

The C/N ratios of elephant nails are extraordinarily high. The reason for this phenomenon is unknown. It would be interesting to investigate why elephant nails show much higher C/N ratios than nails from other organisms. Is this also the case for other elephant tissues? Since the C/N ratio indicates the quality and reliability of a sample used for stable isotope measurements, it is important to examine the reason for the enrichment shown in elephant nails.

- *How can the different $\delta^{15}N$ values in animals from different zoos be explained?*

These differences in $\delta^{15}N$ values of animals from different zoos are considerably small, perhaps reflecting variance in the feed at different zoos. Are these small differences in diets large enough to be traced back in the animal tissues?

- *To what extent are $\delta^{15}N$ values of elephants influenced by water and food stress?*

Since elephants achieve lower digestion coefficients than horses and rhinoceroses, they might experience more stress during periods of water and food shortage compared to these other herbivores. In these circumstances, their nitrogen balance would be more negative than for horses and rhinoceroses, which might be an explanation for the enriched $\delta^{15}N$ values of wild elephants, and probably Pleistocene mammoths relative to other herbivores. One would need to measure the $\delta^{15}N$ values in tissues with a short turnover time (e.g. nail or hair) of modern elephants, who endure situations of water and food sufficiency alternated with situations of scarcity. Such measurements are possible by means of controlled-feeding studies, if food and water stress can be taken into account, but there would be serious ethical constrains for such research. Alternatively, the $\delta^{15}N$ values in tissues of multiple wild elephant could be measured. Such research needs to be performed to gain more insight into the part of water and food stress in the determination of $\delta^{15}N$ values.

Research on the questions mentioned above will enlarge our understanding of fossil data. Since results from the present study show that comparison of Pleistocene mammals with their modern analogues is possible and can give insight into the factors that determine stable isotope ratios, it is important that more stable isotope values from fossil material

will be investigated and explained by means of studying stable isotope ratios in modern analogues. I am convinced that the results of this present study trigger future research.

Acknowledgements

The authors' thanks are due to Prof.dr. M. van Kolfschoten and Prof. dr. J. van der Plicht for their assistance and for valuable advice on the first draft of this thesis. Moreover, Prof. dr. J. van der Plicht and the other staff members of the Center for Isotope Research of the University of Groningen are gratefully thanked for their help with sample preparation and measurement. The author is also grateful to the keepers of the elephant, horse, zebra, and rhinoceros and the veterinary surgeons of the participating zoos for their engaged support of this study and for providing nail samples.

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Appendix A $\delta^{15}N$ values of Pleistocene mammoth, horse and rhinoceros

Coelodonta antiquitatis

Age (BP)	"+/-	$\delta^{13}C$	$\delta^{15}N$	Country	Place	Source
±40.000		-20.9	5.5	Belgium	Scladina Cave (1A)	Bocherens 2003
±40.000		-20.3	6.9	Belgium	Scladina Cave (1A)	Bocherens 2003
±40.000		-20	6.4	Belgium	Scladina Cave (1A)	Bocherens 2003
±40.000		-21.1	5.3	Belgium	Scladina Cave (1A)	Bocherens 2003
±40.000		-20.4	7.5	Belgium	Scladina Cave (1A)	Bocherens 2003
±40.000		-20.6	5.5	Belgium	Scladina Cave (1A)	Bocherens 2003
±28-40.000		-20.6	3.7	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-20.6	5.9	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-20.6	4.4	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-22.5	6.7	Great-Britain	Kent's Cavern	Bocherens 2003
32290	190	-20.6	6.8	Germany	Sachsen, Duna, Herzberg-Osterode	CIO, GrA-32598 (D. Mol)
±20.000		-20.6	8.4	Russia	Yakutia, Tchouraptcha	Bocherens 2003

Equus caballus

Age (BP)	"+/-	$\delta^{13}C$	$\delta^{15}N$	Country	Place	Source
±40.000		-21.7	5.2	Belgium	Scladina Cave	Bocherens 2003
±40.000		-21.7	5.1	Belgium	Scladina Cave	Bocherens 2003
±40.000		-21.9	5	Belgium	Scladina Cave	Bocherens 2003
±40.000		-21.5	4.8	Belgium	Scladina Cave	Bocherens 2003
±40.000		-21.6	7	Belgium	Scladina Cave	Bocherens 2003
±40-45.000		-20.1	4.9	France	Marillac	Bocherens 2003
±40-45.000		-20.7	4.8	France	Marillac	Bocherens 2003
±40-45.000		-20.8	3	France	Marillac	Bocherens 2003
±40-45.000		-20.5	3	France	Marillac	Bocherens 2003
±40-45.000		-20.5	5	France	Marillac	Bocherens 2003

***Equus caballus* (continued)**

Age (BP)	"+/-	$\delta^{13}C$	$\delta^{15}N$	Country	Place	Source
±40-45.000		-21.0	5.1	France	Marillac	Bocherens 2003
±40-45.000		-20.5	4.8	France	Marillac	Bocherens 2003
±40-45.000		-20.8	4.8	France	Marillac	Bocherens 2003
±40-45.000		-20.3	3.6	France	Marillac	Bocherens 2003
±40-45.000		-20.3	4.6	France	Marillac	Bocherens 2003
±40-45.000		-21.9	7.9	France	Marillac	Bocherens 2003
±40-45.000		-20.7	6.8	France	Marillac	Bocherens 2003
±40-45.000		-20.4	5.5	France	Marillac	Bocherens 2003
±40-45.000		-20.7	5.9	France	Marillac	Bocherens 2003
±40-45.000		-22.2	6.4	France	Marillac	Bocherens 2003
±40-45.000		-20.4	7.4	France	Marillac	Bocherens 2003
±40-45.000		-19.2	3.9	France	Marillac	Bocherens 2003
±40-45.000		-20.6	5.3	France	Marillac	Bocherens 2003
±40-45.000		-21.4	6.3	France	Marillac	Bocherens 2003
±40-45.000		-20.4	2.3	France	Marillac	Bocherens 2003
±40-45.000		-20.4	5.5	France	Marillac	Bocherens 2003
±40-45.000		-20.1	4.2	France	Marillac	Bocherens 2003
±30-32.000		-20.6	5.6	France	Saint-Césaire	Bocherens 2003
±30-32.000		-21.2	6.5	France	Saint-Césaire	Bocherens 2003
±30-32.000		-20.3	8.5	France	Saint-Césaire	Bocherens 2003
±30-32.000		-21.2	6.2	France	Saint-Césaire	Bocherens 2003
±36.000		-21.7	5.7	France	Saint-Césaire	Bocherens 2003
±28-40.000		-20.8	4.4	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-21.2	7.8	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-21.2	8.4	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-21.6	6.8	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-21.6	7.7	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-21.9	6.9	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-21.6	6.0	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-21.4	3.3	Great-Britain	Kent's Cavern	Bocherens 2003

***Equus caballus* (continued)**

Age (BP)	"+/-	$\delta^{13}C$	$\delta^{15}N$	Country	Place	Source
±28-40.000		-21.0	8.5	Great-Britain	Kent's Cavern	Bocherens 2003
±28-40.000		-21.6	5.4	Great-Britain	Kent's Cavern	Bocherens 2003
44780	1920	-21.4	5.4	North Sea		CIO, GrA-23582 (J.C. Glimmerveen)
±25.000		-21.1	4.3	Russia	Yakutia, Maksounuocha River	Bocherens 2003
Upper Pleistocene		-22.7	6.8	Russia	Yakutia	Bocherens 2003
18090	80	-21.2	6.3	Russia	Taimyr	CIO, GrA-17351 (D. Mol)
38750	1050	-22.3	5.3	Russia	Taimyr	CIO, GrA-19235 (D. Mol)
22740	170	-20.8	6.0	Russia	Taimyr	CIO, GrA-19236 (D. Mol)

Mammuthus primigenius

Age (BP)	"+/-	$\delta^{13}C$	$\delta^{15}N$	Country	Place	Source
±40.000		-20.9	8.4	Belgium	Scladina Cave (1A)	Bocherens 2003
±40.000		-21.5	9.4	Belgium	Scladina Cave (1A)	Bocherens 2003
±40.000		-21.6	8.3	Belgium	Scladina Cave (1A)	Bocherens 2003
Upper Pleistocene		-20.8	6.4	Alaska	Lost Chicken Creek	Bocherens 2003
Upper Pleistocene		-21.0	6.4	Alaska	Chatanika	Bocherens 2003
Upper Pleistocene		-20.9	6.9	Alaska	Fairbanks	Bocherens 2003
Upper Pleistocene		-21.7	6.8	Alaska	?	Bocherens 2003
Upper Pleistocene		-21.2	8.5	Alaska	Eielson AFB	Bocherens 2003
Upper Pleistocene		-21.1	8.3	Alaska	?	Bocherens 2003
Upper Pleistocene		-20.7	7.2	Russia	Brianskaya Oblast	Bocherens 2003
Upper Pleistocene		-21.7	7.7	Russia	Novo-Petroskoye	Bocherens 2003
±30.000		-22.4	9.1	Russia	Yakutia, Dien-Urech River	Bocherens 2003
±15.000		-20.7	8.1	Russia	Yakutia, Singnigese-Urech River	Bocherens 2003
Upper Pleistocene		-21.8	10.8	Russia	Yakutia, Tchokourdakh	Bocherens 2003
Upper Pleistocene		-22.8	8.8	Russia	Yakutia, Kien-Ajaan River	Bocherens 2003
Upper Pleistocene		-21.5	10.4	Russia	Yakutia, Tchokourdakh	Bocherens 2003
±50.000		-22.3	10.2	Russia	Yakutia, Bolshoj Ljachovskij Isl.	Bocherens 2003
Upper Pleistocene		-21.6	11.4	Russia	Yakutia	Bocherens 2003
Upper Pleistocene		-21.3	7.2	Russia	Yakutia	Bocherens 2003

***Mammuthus primigenius* (continued)**

Age (BP)	"+/-	$\delta^{13}C$	$\delta^{15}N$	Country	Place	Source

20500	90	-21.3	11.2	Russia	Taimyr	CIO, GrA-17347 (D. Mol)
9920	60	-22.8	8.6	Russia	Taimyr	CIO, GrA-17350 (D. Mol)
20950	190	-22.3	8.6	Russia	Taimyr	CIO, GrA-17604 (D. Mol)
41580	1190	-21.8	9.9	Russia	Taimyr	CIO, GrA-17439 (D. Mol)
32180	210	-22.4	9.1	Russia	Taimyr	CIO, GrA-19523 (D. Mol)
14050	70	-21.6	7.2	Russia	Taimyr	CIO, GrA-19310 (D. Mol)
24460	200	-21.6	10.4	Russia	Taimyr	CIO, GrA-19238 (D. Mol)
41200	440	-21.2	6.0	Russia	Taimyr	CIO, GrA-19524 (D. Mol)
24990	150	-22.2	10.1	Russia	Taimyr	CIO, GrA-19311 (D. Mol)
10230	60	-22.0	9.5	Russia	Taimyr	CIO, GrA-19231 (D. Mol)
24980	130	-22.3	11.1	Russia	Taimyr	CIO, GrA-19526 (D. Mol)
24740	150	-21.8	9.7	Russia	Taimyr	CIO, GrA-19273 (D. Mol)
42040	830	-22.5	8.5	Russia	Taimyr	CIO, GrA-25856 (D. Mol)
41300	900	-21.7	8.1	Russia	Yakutia	CIO, GrA-30727 (D. Mol)

Mammuthus species

Age (BP)	"+/-	$\delta^{13}C$	$\delta^{15}N$	Country	Place	Source
12130	80	-20.9	6.2	Canada	Ontario	CIO, GrA-22177 (D. Mol)
12210	80	-20.9	6.2	Canada	Ontario	CIO, GrA-22819 (D. Mol)
28370	200	-21.3	10.8	Russia	Taimyr	CIO, GrA-19275 (D. Mol)
28350	200	-21.8	11.9	Russia	Taimyr	CIO, GrA-19271 (D. Mol)

Appendix B Dietary information of Pleistocene mammoth, horse and rhinoceros

Although the stable nitrogen isotope ratio of mammoths seems to indicate that mammoths were carnivores, it is undisputable that mammoths were true herbivores. As stated in the second chapter, we know that grass formed the staple diet of mammoths, and, moreover, this is also true for Pleistocene horse and rhinoceros that lived on the mammoth steppe.

Carcasses of *Mammuthus primigenius*, *Coelodonta antiquitatis,* and *Equus* species which are discovered in permafrost environments sometimes still remain their soft tissue body parts due to the preserving conditions of the permafrost. It is possible to reconstruct what these individuals ate before they died by means of studying the contents of their intestinal remains (e.g. Guthrie 1990, 256-7; Olivier 1982, 295, 298). Furthermore, the composition of fossilized feaces and the morphology of teeth and intestinal tract can provide information about the trophic level of an animal.

In this chapter an overview of the results of botanical, palynological and molecular analysis on the intestinal tract and feaces contents of Pleistocene mammoth, horse, and rhinoceros will be given.

Examples of animals of which the colon contents or intestinal tract have been preserved are the Shandrin mammoth, the Beresovka mammoth, the Fishhook mammoth, the Yukagir mammoth, the Yuribei mammoth, the Churapachi rhinoceros, and the Selerikan horse. The results of the studies on the colon contents of the animals mentioned above will be discussed below.

Beresovka mammoth

In 1902 the quite well preserved Beresovka mammoth (44,000±3,500 BP) was excavated near the Beresovka River, a tributary from the Kolyma River. Reconstructions of its diet are based on a chunk of food that was left between its teeth and on analysis of its stomach contents. He fed mainly on grasses (97.1%), the rest of his diet consisted of sedges and mosses. The Beresovka mammoth died during the summer (Vereshchagin *et al*. 1982, 269; Kubiak 1982, 287; Olivier 1982, 297; Guthrie 1990, 4, 258; Ukraintseva 1993, 153).

Shandrin mammoth

The Shandrin mammoth is a 40,350±880 BP old carcass of a woolly mammoth found near the Shandrin River. The gastrointestinal tract of this Shandrin mammoth was virtually intact, so intensive investigation on its contents has been performed. Palynological spectra indicated that the Shandrin mammoth's intestines contained mainly spores of mosses (77.0%). However, this percentage does not give a proper impression of the diet of the animal. After averaging the percentages of pollen and spores, it becomes clear that the staple diet consisted for 80-90%[4] of grasses and sedges, so mosses and woody plants were only present in small numbers. This mammoth possibly passed away in the second half of summer (Guthrie 1990, 27-8; Ukraintseva 1993, 73, 80; Olivier 1982, 297).

Fishhook mammoth

In 1990 a woolly mammoth carcass from 20,620±70 BP was found in the estuary of the Upper Taimyra River. The contents of parts of the stomach and intestinal tract have been preserved. Palynological study on these contents showed a presence of herbaceous plants only, with a very high number (97.8%) of grasses (*Poaceae*). Several unripe grass pollen may indicate that the mammoth died during the flowering season of grasses (Mol *et al*. 2006, 190-1).

Yukagir mammoth

In 2002 the ca. 22,500 cal yr BP old carcass of the Yukagir mammoth was discovered near the Maxunuokha River in northern Yakutia. Palynological investigation of the contents of the lower intestinal tract shows that *Poaceae* (70.6%) and *Asteraceae* (16.4%) formed an important component of the mammoth's last meal. Tree pollen were absent. Amongst the plant macrofossils twigs of dwarf willow (*Salix sp*.) were dominant, but grasses and other herbaceous plants formed a major component as well. Also remains of mosses are identified. Results of DNA analysis performed on the intestinal tract contents

[4] Botanical percentages can vary heavily, because the use different botanical methods (e.g. based on macroremains, pollen, and spores) can lead to different outcomes. Moreover, the contents of different body parts (e.g. stomach, large intestine, and rectum) show dissimilarities in composition.

of the Yukagir mammoth confirm the presence of *Salix* and *Asteraceae*. The mammoth probably died between late autumn and early spring (Van Geel *et al*. 2008, 361, 366-9).

Yuribei mammoth

Palynological study on the stomach and colon contents of the Yuribei mammoth shows a predominance of herbaceous plants, with percentages of 45.3% and 62.9% respectively. Amongst those herbaceous plants is grass (*Poaceae*) the most common, followed by sedge (*Cypersceae*) (Ukraintseva 1993, 113-4). The Yuribei mammoth owes its name to the Yuribei River where it died 9,730+/-300 BP (Ukraintseva 1993, 153[5]; Van Geel *et al*. 2008, 373).

Bechan Cave, North America

In the dry Bechan Cave in Southern Utah (Colorado Plateau), large mammoth dung boluses have been recovered, ranging in age from 11,670 to 13,505 years ago. These feaces had been studied for their contents; they appeared to be composed for the most part of grasses and sedges with a maximum of 95%. A number of the boluses contained some remains of woody plant remains (Mead *et al*. 1986, 121, 124).

Churapachi rhinoceros

Near Churapachi (Yakutia) a carcass of an old female woolly rhinoceros was discovered in 1972. Although the major part of the body's soft tissues had rotten away, the gastrointestinal tract has been preserved. Palynological analysis of the gastrointestinal tract contents presented a predominance of grasses (89%). The rest consisted for the most part of *Asteraceae* (7.0%). Macroremains found in the area of the large intestine comprised shoots of grasses and sedges, amongst which cottongrass (Vereshchagin *et al*. 1982, 271; Guthrie 1990, 34).

Selerikan horse

[5] On page 108 of the same book Ukraintseva indicates another date for the Yuribei mammoth, namely 9,730+/-300 BP (Ukraintseva 1993, 153).

In 1968 the carcass of a 37,000 +/- 2000 BP old stallion was discovered in Indigirka River Basin near Selerikan. Fortunately, its gastrointestinal tract was well-preserved. Botanical and palynological investigation showed a majority of herbaceous plants (90%), of which festucoid grasses and sedges predominated. Furthermore, 5-7% of the intestinal contents consisted of woody plants (mainly *Salix* and *Betula nana*) and mosses represented 1-2%. The season in which the horse died is summer, more precisely late July or early August (Guthrie 1990, 30, 33; Vereshchagin *et al*. 1982, 270-1; Ukraintseva 1993, 84, 98).

The dietary patterns of the animals mentioned above show that mammoths, woolly rhinoceroses and Pleistocene horses had a preference for grass and other herbaceous plants. At least, this is true during the warm seasons. The individuals discussed above, with exception of the Yukagir mammoth, probably all died in summer. This is why we hardly know anything of the winter food of these species. During summer, the browsing fraction is low as opposed to the grazing fraction. In winter, on the other hand, browsing might have been playing a far more important role (Putshkov 2003, 369). The diet of the Yukagir mammoth, whose death is estimated to have been in the period between summer and winter, does not demonstrate this: grasses and herbaceous plants dominated like in summer diets.

Appendix C Dietary data of modern animals

Diet of elephants from different zoos per day

Elephant	Zoo Beeksebergen	Zoo Emmen	Zoo Amersfoort	Zoo Rotterdam	Zoo Eindhoven
Grass (fresh)	√	100 kg			summer
Hay/meadow			60 kg	20-30 kg	60 kg
Branches/twigs	cortex/leaves/branches	cortex/leaves	willow/birch/beech/oak	willow (summer)	4 branches
Fruit	√	2 kg	4 kg	2 kg	4 kg
Vegetables	√	2 kg	4 kg	2 kg	7 kg
Maize			20 corncobs		
Bread		3 kg		0.8 kg	
Horse/pachyderm pellet	4 kg	female 5 kg/male 8-10 kg	4 kg	2 kg	3 kg

Diet of Rhinoceros from different zoos per day

Rhinoceros	Zoo Beeksebergen	Zoo Emmen	Zoo Amersfoort	Zoo Rotterdam
Grass	Ad lib	ad lib (+/- 40 kg)		
Hay/meadow	?		30 kg	20 kg
Branches/twigs	?		branches	
Fruit	?	male 0.75 kg	0.5 kg	5 kg
Vegetables	?	male 1.25 kg	1 kg	6.2-6.6 kg
Maize	?		10 corncobs	
Bread	?	0.4 kg		1.6 kg
Horse /pachyderm pellet	4 kg (	6 kg	4 kg	3 kg

Diet of zebra from Zoo Emmen and horse from Riding school Leiden per day

Horse	Zoo Emmen	Riding School Leiden
Grass	ad lib	ad lib
Hay/meadow	ad lib	
Branches/twigs		
Fruit		
Vegetables		
Maize		
Bread		
Horse/pachyderm pellet	female 0.5/male 1 kg	2-6 kg

Most important ingredients of horse and pachyderm pellets. A=Hippostar HORSE QUICK: zebras in Zoo Emmen; B=Van Cooten Diervoeders Lienden: elephants/rhinoceroses in Zoo Emmen; C=pachyderm pellet (CODE: 5820 8mm): elephants in Zoo Amersfoort; D=Hippostar PERFORMANCE: rhinoceroses in Zoo Amersfoort; E= Horse pellet PERFORMANCE 2: elephants in Zoo Rotterdam; F= Horse pellet BLIJDORP NR 2215506: rhinoceroses in Zoo Rotterdam; G= Vente Diervoeding horse pellet SUPPORT: horses in Riding school Leiden; H=Kasper faunafood ELEPHANT CUBES (6318): elephants in Zoo BeekseBergen; I=Hippostar MARE&FOAL (1342): rhinoceroses in Zoo BeekseBergen

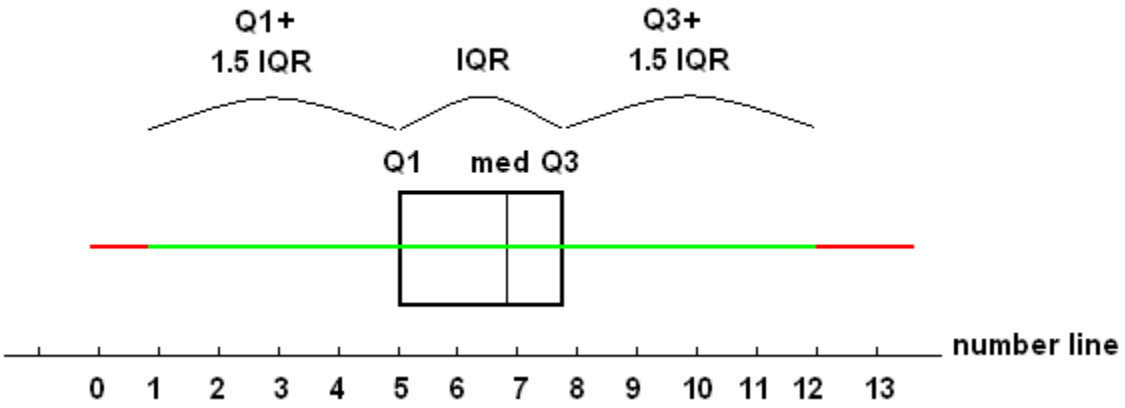
	A	B	C	D	E	F	G	H	I
Protein	7.5%			8.5%	9.65%	14.56%			
Crude protein	10.7%	15%	166.38g/kg	11.7%			11.9%	16.0%	15.0%
Crude fat	2.5%	7.8%	44.89g/kg	4.5%	3.02%	3.90%	2.8%	5%	4.0%
Crude fibre	9.0%	10%	101.17g/kg	4.5%			9.1%	9%	5.5%
Crude ash	7.2%	13%	134.03g/kg	8.0%	10.52%	8.00%	6.9%	14%	8.5%
Ca	9.0g/kg	1.5%	15g/kg	10.0g/kg	0.59%	0.73%	10.0%	1.62%	1.37%
P	4.0g/kg	0.5%	3.26+6.5 g/kg	4.7g/kg	0.26%	0.49%	5.1%	0.65%	0.55%
Mg	3.7 g/kg		3.98 g/kg	4.3g/kg			2.9g/kg	0.40%	0.48%
Na	4.1 g/kg		18 g/kg	7.3g/kg			4.1g/kg	1.77%	0.45%
Vit A	14,200 IU/kg	32,000 IU	30,000 IU/kg	18,800 IU/kg	2.25 IU/g	9.97 IU/g	14,000 IU/kg	30,000 IU/kg	28,000 IU/kg
Vit D3	2,525 IU/kg	7,400 IU	6,000 IU/kg	3,325 IU/kg	0.42 IU/g	2.23 IU/g	2,000 IU/kg	6,000 IU/kg	4,200 IU/kg
Vit E	150 mg/kg	400 mg/kg	500 mg/kg	500 mg/kg	161.32 IU/kg	166.31 IU/kg	150 IU/kg	195 mg/kg	200 mg/kg
Biotine (mcg/kg)	760	5000	5000	1000			200	1250	940
Cu (mg/kg)	25		9.98	33			30	10	150

Appendix D Statistics

N.B. In this thesis the word 'sample' generally refers to the material measured of one specific animal. However, in this appendix and in other statistical contents in this thesis 'sample' refers to all values of one specific species group, e.g. all values of elephants. Here, the value of one specific animal is called 'observation'.

Outliers

Determining outliers is a subjective process. However, it is important to define a limiting value for each sample. This limiting value contributes to estimate a $\delta^{13}C$ or $\delta^{15}N$ value to be accepted within the sample, or to be an outlier and thus to be rejected.
In this research, for determining outliers the interquartile range of the samples is used.



Boxplot example

In the figure above, a boxplot of a made-up data range is illustrated. In this example, the lower quartile (Q1) is 5 and the upper quartile is 7.8. The interquartile rang (IQR) is derived by extracting Q1 from Q3: 7.8-5=2.8.
Outliers are these samples, which have a lower value than Q1-1.5 IQR or Q3+1.5 IQR. In this example, 1.5 IQR=4.2. Q1-1.5 IQR=0.8 and Q3+1.5 IQR=12. Samples with a value lower than 0.8 or higher than 12 are outliers and should be rejected. Samples with values between 0.8 and 12 are accepted (Fletcher *et al*. 2001, 47-9).

Nitrogen

Elephants

Q1	5.98
median	6.51
Q3	6.95
Q3-Q1	0.97
1.5 x 0.9725	1.46
Q1 - 1.46	4.52
Q3 + 1.46	8.41

Accepted: >4.52 <8.41
Rejected: none

Horses

Q1	7.30
median	7.54
Q3	7.76
Q3-Q1	0.46
1.5 x 0.455	0.68
Q1 - 0.6825	6.62
Q3 + 0.6825	8.44

Accepted: >6.62 <8.44
Rejected: 9.81
Outliers: Paard - Lobke 3 (-26.17; 9.81)

Rhinoceroses

Q1	6.02
median	6.31
Q3	6.51
Q3-Q1	0.49
1.5 x 0.49125	0.74
Q1 -0.736875	5.28
Q3 + 0.736875	7.25

Accepted: >5.28
Rejected: none

Carbon

Elephants

Q1	-26.85
median	-26.36
Q3	-26.02
Q3-Q1	0.83
1.5 x 0.83	1.25
Q1 - 1.245	-28.10
Q3 + 1.245	-24.78

Accepted: >-28.10<-24.78
Rejected: none

Horses

Q1	-26.20
median	-25.99
Q3	-25.55
Q3-Q1	0.47
1.5 x	0.70
Q1 - 0.6975	-26.89
Q3 + 0.6975	-24.85

Accepted: >-26.89<-24.85
Rejected: -23.68
Outliers: Paard - Mike 2 (-23.68; 7.75)

Rhinoceroses

Q1	-25.92
median	-25.51
Q3	-25.28
Q3-Q1	0.63
1.5 x	0.95
Q1 - 0.94875	-26.86
Q3 - 0.94875	-24.33

Accepted: >-26.86<-24.33
Rejected: -23
Outliers: Neushoorn Beeksebergen (-23; 6.30)

Mann-Whitney U test

The Mann-Whitney U test is a test of difference. The null hypothesis in the Mann–Whitney test is that the probability distributions of the two samples under investigation are equal. The alternative hypothesis is that the probability distributions of the two samples differ significantly.

First the statistic, called U, should be calculated as follows:

1. Rank the values ($\delta^{15}N$ or $\delta^{13}C$) in order of size.
2. Choose the animal species (elephant, horse or rhinoceros) with the smaller number of values.
3. Take each observation in the smallest sample and count the number of observations in the larger sample that have a lower value than it.
4. Total these counts: the sum is the U statistic. Calculate the converse of the U statistic (U') with the formula: $U'=n_1n_2-U$, in which n_1=size of the smallest sample and n_2=size of the largest sample. It is the smaller of these (U or U') that is used in the test.
5. Look up the critical value in table below.
6. Determine if the U or U' value is smaller than the critical value (smaller= H_1; larger is H_0) (Shennan 1997, 65-70).

Abbreviations used:

E=elephant

H=horse

R=rhinoceros

n= sample size (number of observations in sample)

n_1=size of the smallest sample

n_2=size of the largest sample

H_0=null hypothesis (no difference)

H_1=alternative of null hypothesis (difference)

Nitrogen

Horse/elephant

Smallest sample=elephant

	Species	$\delta^{15}N$
	H	8.390
	H	8.240
	H	8.085
15	E	7.935
15	E	7.918
15	E	7.808
	H	7.765
	H	7.750
	H	7.625
	H	7.605
	H	7.555
	H	7.525
	H	7.475
	H	7.470
	H	7.415
	H	7.300
	H	7.295
4	E	6.948
	H	6.900
	H	6.810
2	E	6.573
2	E	6.553
2	E	6.510
2	E	6.418
2	E	6.335
2	E	5.975
2	E	5.920
	H	5.825
1	E	5.815
1	E	5.628
	H	5.610
65		

U'=19 x 13 – 65
U'=247 - 65=182

Critical value=72

65<72, so H_1

Horse/rhinoceros

Smallest sample=rhinoceros

	Species	$\delta^{15}N$
	H	8.390
	H	8.240
	H	8.085
	H	7.765
	H	7.750
	H	7.625
	H	7.605
	H	7.555
	H	7.525
	H	7.475
	H	7.470
	H	7.415
	H	7.300
	H	7.295
4	R	7.105
	H	6.90
	H	6.810
2	R	6.570
2	R	6.325
2	R	5.925
	H	5.825
	H	5.610
0	R	5.555
10		

U'=19 x 5 – 10
U'=95 - 10=85

Critical value=19

10<19, so H_1

Elephant/rhinoceros

Smallest sample=rhinoceros

	Species	$\delta^{15}N$
	E	7.935
	E	7.918
	E	7.808
10	R	7.105
	E	6.948
	E	6.573
8	R	6.570
	E	6.553
	E	6.510
	E	6.418
	E	6.335
4	R	6.325
	E	5.975
3	R	5.925
	E	5.920
	E	5.815
	E	5.628
0	R	5.555
25		

U'=13 x 5 – 25
U'=65 - 25=40

Critical value=12

25>12, so H_0

Carbon

Horse/elephant

Smallest sample=elephant

	H	-24.96
	H	-25.15
	H	-25.27
	H	-25.49
	H	-25.57
14	E	-25.75
	H	-25.82
	H	-25.86
	H	-25.91
11	E	-25.93
	H	-25.96
	H	-26.01
9	E	-26.02
9	E	-26.02
	H	-26.05
	H	-26.06
7	E	-26.13
	H	-26.16
	H	-26.17
	H	-26.27
4	E	-26.32
4	E	-26.36
	H	-26.41
3	E	-26.42
3	E	-26.53
	H	-26.58
	H	-26.68
1	E	-26.85
	H	-26.88
0	E	-26.91
0	E	-27.07
0	E	-27.07
65		

U'=13 x 19 – 65
U'=247 - 65=182

Critical value=72

65<72, so H_1

Horse/rhinoceros

Smallest sample=rhinoceros

	H	-24.96
	H	-25.15
17	R	-25.22
	H	-25.27
16	R	-25.47
	H	-25.49
15	R	-25.54
	H	-25.57
	H	-25.82
	H	-25.86
	H	-25.91
	H	-25.96
	H	-26.01
9	R	-26.04
	H	-26.05
	H	-26.06
	H	-26.16
	H	-26.17
	H	-26.27
	H	-26.41
	H	-26.58
	H	-26.68
1	R	-26.82
	H	-26.88
58		

U'=5 x 19 – 58
U'=95 - 10=85

Critical value=19

10<19, so H_1

Elephant/rhinoceros

Smallest sample=rhinoceros

13	R	-25.22
13	R	-25.47
13	R	-25.54
	E	-25.75
	E	-25.93
	E	-26.02
	E	-26.02
9	R	-26.04
	E	-26.13
	E	-26.32
	E	-26.36
	E	-26.42
	E	-26.53
4	R	-26.82
	E	-26.85
	E	-26.91
	E	-27.07
	E	-27.07
52		

U'=5 x 13 – 52
U'=65 - 52=13

Critical value=12

52>12, so H_0

Critical Values for the Mann-Whitney U-Test

Level of significance: 5% (P = 0.05)

Size of the smallest sample (n_1) \ Size of the largest sample (n_2)	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
3	0	1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9	10	10	11	11	12	13	13
4	1	2	3	4	4	5	6	7	8	9	10	11	11	12	13	14	15	16	17	17	18	19	20	21	22	23
5	2	3	5	6	7	8	9	11	12	13	14	15	17	18	19	20	22	23	24	25	27	28	29	30	32	33
6		5	6	8	10	11	13	14	16	17	19	21	22	24	25	27	29	30	32	33	35	37	38	40	42	43
7			8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54
8				13	15	17	19	22	24	26	29	31	34	36	38	41	43	45	48	50	53	55	57	60	62	65
9					17	20	23	26	28	31	34	37	39	42	45	48	50	53	56	59	62	64	67	70	73	76
10						23	26	29	33	36	39	42	45	48	52	55	58	61	64	67	71	74	77	80	83	87
11							30	33	37	40	44	47	51	55	58	62	65	69	73	76	80	83	87	90	94	98
12								37	41	45	49	53	57	61	65	69	73	77	81	85	89	93	97	101	105	109
13									45	50	54	59	63	67	72	76	80	85	89	94	98	102	107	111	116	120
14										55	59	64	67	74	78	83	88	93	98	102	107	112	118	122	127	131
15											64	70	75	80	85	90	96	101	106	111	117	122	125	132	138	143
16												75	81	86	92	98	103	109	115	120	126	132	138	143	149	154
17													87	93	99	105	111	117	123	129	135	141	147	154	160	166
18														99	106	112	119	125	132	138	145	151	158	164	171	177
19															113	119	126	133	140	147	154	161	168	175	182	189
20																127	134	141	149	156	163	171	178	186	193	200
21																	142	150	157	165	173	181	188	196	204	212
22																		158	166	174	182	191	199	207	215	223
23																			175	183	192	200	209	218	226	235
24																				192	201	210	219	228	238	247
25																					211	220	230	239	249	258
26																						230	240	250	260	270
27																							250	261	271	282
28																								272	282	293
29																									294	305
30																										317

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