Stable isotopes on forensic cold cases from the Greater Cape Town area

by

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Abstract

Stable isotope analysis is an increasingly significant approach in forensic science because it offers a way of obtaining information about the origins and diets of unidentified human remains discovered in forensic settings. This study investigates forensic cold cases from the Greater Cape Town area, to determine the range of variation in stable carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), oxygen ($\delta^{18}O$), and strontium ($^{87}Sr/^{86}Sr$) isotopes in the city's very diverse population. The project selected 37 individuals from the Human Skeletal Repository in the Department of Human Biology at the UCT Medical School. Small samples were taken from teeth (dentine collagen and enamel) and ribs (bone collagen), to investigate diets and likely place of residence during early childhood (as recorded in the isotopic composition of teeth) and in adulthood (as recorded in ribs). Carbon, nitrogen, and strontium isotope ratios show very high diversity, oxygen isotopes less so. $\delta^{13}C$ values reflect a range of diets, with most individuals having consumed mixed C_4-C_3-based diets. A few consumed C_4-based diets during early life, shifting towards a more C_3-based diet in later life. This pattern is consistent with moving from rural areas of South Africa to Cape Town. $^{87}Sr/^{86}Sr$ values were all compatible with the Greater Cape Town area, although other regions have similar values. One important finding of this study is that intra-individual, inter-tissue variation in $\delta^{13}C$ and $\delta^{15}N$ can be higher than 2‰. These can shed light on changes during an individual's life, such as dietary changes associated with transitioning from rural to urban areas or altering protein sources. Thresholds for assessing the number of individuals in co-mingled human remains may need to be reassessed in the light of these results. In the long term, these results may be useful in larger-scale projects using isotopes as part of a suite of tools to determine the region/s of origin of unidentified deceased migrants and displaced children in Africa.

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CHAPTER ONE: INTRODUCTION

1.1. Background

Over the past years, isotope analysis has grown in significance as a technique in the field of forensic sciences (Jørkov *et al*., 2009, Bartelink & Chesson, 2019). It provides novel ways of determining the diets and origins of unidentified human remains found in forensic contexts, (Schwarcz, 2000). According to Bartelink *et al.,* (2014), stable isotope ratios present in human tissues serve as a record of the food eaten throughout one's life, as well as the place where it was produced. By examining multiple isotopes, additional evidence can be gathered, such as where the individual was born, where they lived long-term as an adult, and their food preferences (depending on the available tissues for examination, e.g., bone, teeth, hair, and nails), (Schoeninger & DeNiro 1984). Stable isotope analysis is a valuable means of estimating geographical (or geological) origin, as well as gathering information about diet, with the potential to provide helpful clues in forensic situations, (Hedges *et al*., 2007).

This study is a multi-stable isotope approach, assessing the isotopic evidence from (n=37) forensic cold cases found in the Greater Cape Town area. It focuses on four isotope pairs, i.e., $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{87}Sr/^{86}Sr$. Stable carbon and nitrogen isotope ratios in collagen and carbon in bioapatite are indicators of food consumption habits, which frequently fluctuate between geographical areas as a result of cultural dietary variations. Oxygen and strontium isotopes, on the other hand, can provide useful information on geographical region of origin and long-term residence.

Carbon isotope ratios depend primarily on the photosynthetic pathway (C_3 or C_4) of plants at the base of the food web (van der Merwe 1982). In the southern African context, we see more positive $\delta^{13}C$ values in people who consume large quantities of maize-based [C_4] foods compared with those reliant mainly on wheat/rice/oats-based [C_3] foods. Nitrogen isotope ($\delta^{15}N$) values depend upon the proportions of plant and animal foods in the diet, and are more positive in hotter, drier climates (Ambrose 1991) and in marine food consumers (Schoeninger et al., 1983; Schoeninger & DeNiro, 1984). Oxygen isotope ($\delta^{18}O$) values are determined mainly by drinking water and to a lesser extent by food. Across Africa, $\delta^{18}O$ values are higher in hotter, drier climates, with some of the highest values in the world coming from the Horn of Africa (Bowen, 2010). $^{87}Sr/^{86}Sr$ ratios reflect the geological substrate on which the person lived, and/or from which their food derives. In urban populations relying on commercially sourced food, $^{87}Sr/^{86}Sr$ is likely to be less useful, but individuals who spent their early lives as subsistence farmers will have $^{87}Sr/^{86}Sr$ in their tooth enamel reflecting the geological substrate on which they lived. These isotopic indicators therefore tell us about region of origin, not specific localities, but use of multiple isotopes improves discriminatory power (Lee-Thorp, 2008; O'Connell, 2023).

This study is an observational, retrospective study based on the remains of individuals curated in the Human Skeletal Repository in the Department of Human Biology, University of Cape Town. Analysis of teeth will reflect diet and place of residence at the time of tissue formation (Beaumont & Montgomery, 2015). Analysis of bone, which continues to remodel after completion of growth, provides a longer-term average of diet and residency, (Katzenberg & Lovell, 1999; Beaumont & Montgomery, 2015). Substantial differences between the two raise the possibility of major changes in lifestyle or place of residence, as in migration from one region to another. This can be further investigated by analysis of additional samples formed at intermediate periods of the person's life.

1.2. Research Aims

The aim of this study is to document the isotopic variation seen in the population of the Greater Cape Town area, as a first step towards evaluating how useful forensic isotopes are likely to be in this region and southern Africa at large. Specific goals are:

- To determine the dietary patterns of these individuals during life
- To assess the degree of isotopic variation among the group
- To explore the extent of within-individual isotopic variation that might indicate a change in place of residence, i.e., moving to Cape Town
- If possible, contribute towards the identification of some of the selected individuals.
- In the long term, these results may be useful in larger-scale projects using isotopes as part of a suite of tools to determine the region/s of origin of unidentified deceased migrants and displaced children in Africa.

1.3. Dissertation Outline

The dissertation comprises five chapters, of which this introduction is the first. Chapter Two is the literature review. This will give an overview of the basic principles of stable isotopes as dietary and environmental tracers, briefly describe the formation and ages of development of the different skeletal parts that have been sampled, and lastly outline some of the stable isotope studies on human skeletal remains which have been conducted before, both in South Africa and further afield. Chapter Three describes the materials and methods for the study, i.e., the sampling strategy, how different skeletal elements were sampled, and the methods employed to determine the various isotopic ratios. Chapter Four presents the results of the analyses. Chapter Five is the Discussion and Conclusion, which will evaluate the results of this study in the light of previously published work. It will also summarize the main findings of the study and make recommendations for future research.

CHAPTER TWO: BACKGROUND AND LITERATURE REVIEW.

2.1. Stable Isotopes: An Overview

Isotopes are naturally occurring forms of an element with the same number of protons and electrons but different nuclear masses due to differences in the number of neutrons (Sharp 2007). They respond at different rates to chemical reactions due to variances in their kinetic and thermodynamic properties caused by their disparate masses. As a result, they exhibit regular patterns throughout the lithosphere and biosphere. Isotopes can be stable or radioactive, for example, the carbon element has three principal isotopes, two stable (^{12}C and ^{13}C) and the other radioactive (^{14}C). While unstable (radioactive) isotopes decay radioactively at predictable and quantifiable rates, the abundances of stable isotopes remain constant over time (Hoefs & Hoefs 1987).

There are two or more stable isotopes for most elements. More than 300 stable isotopes have been identified; the lighter isotope is more prevalent for each element (Sulzman 2007).

During chemical reactions, heavier isotopes react more slowly than lighter ones because they form stronger bonds that require more energy to break (Schwarcz & Schoeninger 1991). The products of a chemical reaction, therefore, have different proportions of heavy and light isotopes compared with the reactants. This shift is known as 'fractionation.' As a result, isotopes of the same element are distributed through the biosphere and the geosphere in patterned ways that enable the tracing of many biological and geological activities (Schwarcz & Schoeninger 1991; Lee-Thorp 2008). Dietary tracing has, to date, focused mainly on carbon, nitrogen, hydrogen, oxygen, sulfur, and strontium isotopes, because these elements are important constituents of living tissues, so can help track the food which was consumed as well as the origins of the individuals.

The delta (δ) notation is used to express isotopic abundance. This notation compares a sample's ratio (R) of a heavier to a lighter isotope with that of an internationally recognized standard (Slater *et al.*, 2001). The permil (‰) notation denotes the units, in parts per thousand, used when expressing a delta (δ) value. The equation for δ is:

$$\delta(‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$$

Standards are internationally accepted reference materials. The reference material for carbon is Belemnite from the Pee Dee formation (PDB), a marine limestone or VPDB, introduced subsequently when the original PDB was no longer available. The $^{13}C/^{12}C$ ratio of VPDB is the same as PDB. On the other hand, the reference material for nitrogen is atmospheric nitrogen (AIR) (Hayes 1983). Standards have delta values of 0‰. The carbon in most biological organisms contains a lower proportion of ^{13}C than PDB, hence $\delta^{13}C$ values are frequently negative (Ambrose 1993).

2.1.1. Stable Carbon Isotopes

Carbon is one of the most essential chemical elements as it is a primary component of proteins, nucleic acids, lipids, and carbohydrates. There are two stable isotopes of carbon, ^{12}C, and ^{13}C, occurring naturally in abundances of 98.89 and 1.11%, respectively. Plants start the carbon cycle by turning atmospheric carbon dioxide (CO_2) into carbohydrates. Atmospheric CO_2 currently has a $\delta^{13}C$ value of approximately -8.5‰ *(Isotopic Data Graphics Gallery Scripps CO2 Program (ucsd.edu)),* this value continues to change as the burning of fossil fuel contributes CO_2 to the atmosphere.

Natural variation in the ratios of stable carbon isotopes in biological systems is due mainly to photosynthesis. There are three main types of photosynthetic pathways used by plants i) the Calvin-Benson cycle (C_3) (Calvin & Benson 1948), ii) the Hatch-Slack cycle (C_4) (Hatch & Slack 1970), and iii) the Crassulacean Acid Metabolism (CAM) cycle (Osmond 1978). The C_3 pathway is mostly used by dicotyledons and some monocotyledons, whereas the C_4 pathway is used mostly by monocotyledons and the CAM pathway is used by xerophytes (Bocherens 1997).

The diffusion of CO_2 molecules via the stomata into the leaf's air-filled spaces is the first step in the C_3 pathway (Kohn & Cerling 2002). This leads to an initial fractionation of about -4.4‰ because of the slower movement of the $^{13}CO_2$ as compared to the $^{12}CO_2$ molecules. CO_2 is then fixed via the ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo) enzyme which favors ^{12}C over ^{13}C. C_3 plants also respire during the night, giving off CO_2, which adds another step in the fractionation process. As a result of all these factors, the mean $\delta^{13}C$ value of C_3 plants is $-28.77 \pm 2.68‰$

(1σ, *n* = 3478) (Cornwell *et al.,* 2017). A wide range of trees and woody shrubs, herbs, vegetables as well as some grasses in temperate environments follow the C_3 photosynthetic pathway.

The Hatch-Slack or C_4 photosynthetic pathway discriminates less against ^{13}C as compared to the C_3 pathway. Firstly, the CO_2 is transferred into the bundle sheath cells after being fixed in the oxaloacetate. In this instance, RuBisCo reabsorpts and refixes CO_2 to stop CO_2 loss. With a mean of −12.90 ± 1.52‰ (1σ, n = 137), C_4 plants have more positive $\delta^{13}C$ values (Cornwell *et al*., 2017). C_4 pathway is mainly found in plants in hot arid climates and tropical grasses such as sorghum, millet, maize and sugarcane. C_4 plants have an advantage over C_3 in conditions of high temperature (Ehleringer *et al.,* 1997).

The Crassulacean Acid Metabolism (CAM) photosynthetic pathway borrows from both the C_3 and C_4 pathways, however, it is mostly linked to the fleshy-leafed succulent plants that have acclimatized to dry environments. Usually, these plants split their photosynthetic processes between day and night cycles. To minimize water loss, they open their stomata at night to absorb CO_2 and reduce evaporation. PEP carboxylase converts CO_2 into C_4 acids, which are stored in meaty leaves, at night. RuBisCo releases the acids during the day and fixes them again when sunlight becomes available. If the conditions are right for C_3 photosynthesis, the $^{13}C/^{12}C$ ratios of CAM plants can be the same as those of C_3 plants; alternatively, they can lie anywhere in the range of C_4 plants or somewhere in between.

The consumption of food results in the incorporation of carbon into consumer (including human) tissues, allowing consumers of C_3 and C_4 plants to be distinguished from one another using $\delta^{13}C$ readings. However, more fractionation occurs when the isotopic ratios in the plants are incorporated into the tissues of the consumers. Except for lipids, which are deficient in ^{13}C, this extra fractionation between dietary sources and human tissues causes consumers' $\delta^{13}C$ values to be more positive than the foods they consumed (DeNiro & Epstein 1977; Ambrose & Norr 1993). The diet-to-tissue fractionation factors depend on animal species, body tissue, age, animal digestive physiology, and the quality of consumed food (Caut *et al.,* 2009; Schoeninger 2014). This fractionation can be determined by two techniques: animal-controlled feeding experiments and the use of measurements from wild animals in environments where the isotopic composition is known. Early work by Lee-Thorp *et al.* (1989: 594) proposed that in humans, the diet to bone collagen fractionation has a $\delta^{13}C$

enrichment factor of +4 to +5‰, which has been broadly supported by more recent studies (Howland *et al.,* 2003; Warinner & Tuross 2009). An individual who consumes a mostly C_3 diet will have a $\delta^{13}C_{bone\ collagen}$ value in the region of -20‰, while a mainly C_4 diet will lead to a value of approximately -8‰. Carbon in hydroxyapatite in human tooth enamel is enriched in ^{13}C by about +10‰ relative to diet (Lee-Thorp, *et al*., 1989; Schoeninger, 2014). Therefore, the $\delta^{13}C_{enamel}$ values for someone who consumes a C_3 diet will be approximately -14‰ (see also Loftus & Sealy, 2012), while for a primarily C_4 diet they may be 0‰ or even slightly positive. The changing baseline of $\delta^{13}C_{atmosphere}$ is a significant complication in estimating these endpoints.

2.1.2. Stable Nitrogen Isotopes

One of the main components of the atmosphere is nitrogen, which is present as N_2 gas. There are two stable isotopes: the most common, ^{14}N, is 99.636%; the rest, 0.364%, is composed of ^{15}N (Berglund & Wieser 2011). Since most living things cannot directly use N2, nitrogen-fixing bacteria must absorb it in order to generate ammonium (NH_4^+), nitrites (NO_2-), and nitrates (NO_3-). The main sources of nitrogen for plants, and consequently for animals, are ammonium and nitrates. During the denitrification process, fixed nitrogen (NO_2-, NO_3-) can also be converted into N_2 gas by bacteria and released back into the environment. The nitrogen isotopes are substantially fractionated by this process, which leaves the soil richer in ^{15}N and preferentially converts ^{14}N to N_2.

The kind of absorbed nitrogen, assimilation processes, and the location of assimilated nitrogen (often in roots or leaves) are among the several variables that affect a plant's nitrogen isotopic composition (Szpak 2014). There is a difference in the $\delta^{15}N$ between mycorrhizal plants and non-mycorrhizal plants. The symbiotic relationship between the mycorrhizae and the plant allows for the mycelium to absorb nutrients from a larger area than the plant itself can access, and exchange some of these for photosynthetic products from the plants (Szpak 2014). Mycorrhizal plants tend to be depleted in ^{15}N because the mycorrhizae retain the heavier isotope (Craine *et al.,* 2009; Hobbie & Högberg, 2012).

Moisture availability also plays a major role in influencing $^{15}N/^{14}N$ ratios in plants and is governed by both meteoric temperature and rainfall (Evans & Ehleringer 1994; Koch 2007). When compared to temperate conditions, plants in dry environments tend to be richer in ^{15}N because of the higher loss of ^{14}N from such systems through denitrification (Hedges *et al.,* 2007; Koch 2007).

In any given food web, values for plants are usually low, and consumers are enriched in ^{15}N in contrast to their food sources (Schoeninger, et al., 1983; Schoeninger & DeNiro 1984; Ambrose 1991; O'Connell *et al.,* 2012). The term 'stepwise enrichment/fractionation' refers to the enrichment process that occurs at every trophic level, with carnivorous animals being more enriched in ^{15}N than plants and animals in the lower trophic levels (See Figure 1). This is the outcome of ^{14}N being eliminated during protein synthesis and ^{15}N being preferentially incorporated (Schoeller 1999). The $\delta^{15}N$ of body tissues is determined primarily by diet (Murphy & Bowman, 2006). The significance of physiological elements that might also aid in the enrichment of ^{15}N in animals is a topic of continuous discussion, particularly when the animals live in arid environments. Ambrose and co-authors argued that in drought-stressed mammals, increased excretion of ^{15}N depleted urea leads to elevated $\delta^{15}N$ in body tissues (Ambrose, 1991, Ambrose & Norr, 1993). Sponheimer *et al.* (2003a), however, argue that when nitrogen fluxes in both urine and faeces are taken into account, this effect becomes less important. Sealy *et al.* (1987) proposed that the gut microbiome effectively adds an additional trophic level, with attendant trophic level fractionation. Sponheimer *et al.* (2003b) have shown that different species of animals eating the same diet can have substantially different diet-to-tissue fractionations (up to 3.6‰). This clearly indicates that, contra Murphy & Bowman (2006), digestive and other processes within animals are important determinants of consumer $\delta^{15}N$ values.

Interpretation of $\delta^{15}N$ values in a forensic context requires consideration of how life stages and socioeconomic factors can affect N values. Fuller *et al.* (2006) showed that $\delta^{15}N$ values change during pregnancy due to alterations in maternal metabolism and nitrogen balance. Nursing infants (who are effectively a trophic level higher than their mothers) have high $\delta^{15}N$ (enriched in by 2 to 3‰ compared to the mother), which drops during weaning (Schurr 1998). Starvation leads to elevated $\delta^{15}N$, as body tissues are catabolized and their constituents re-used (Mekota *et al*., 2006, Beaumont *et al*., 2015, Neuberger *et al*., 2013).

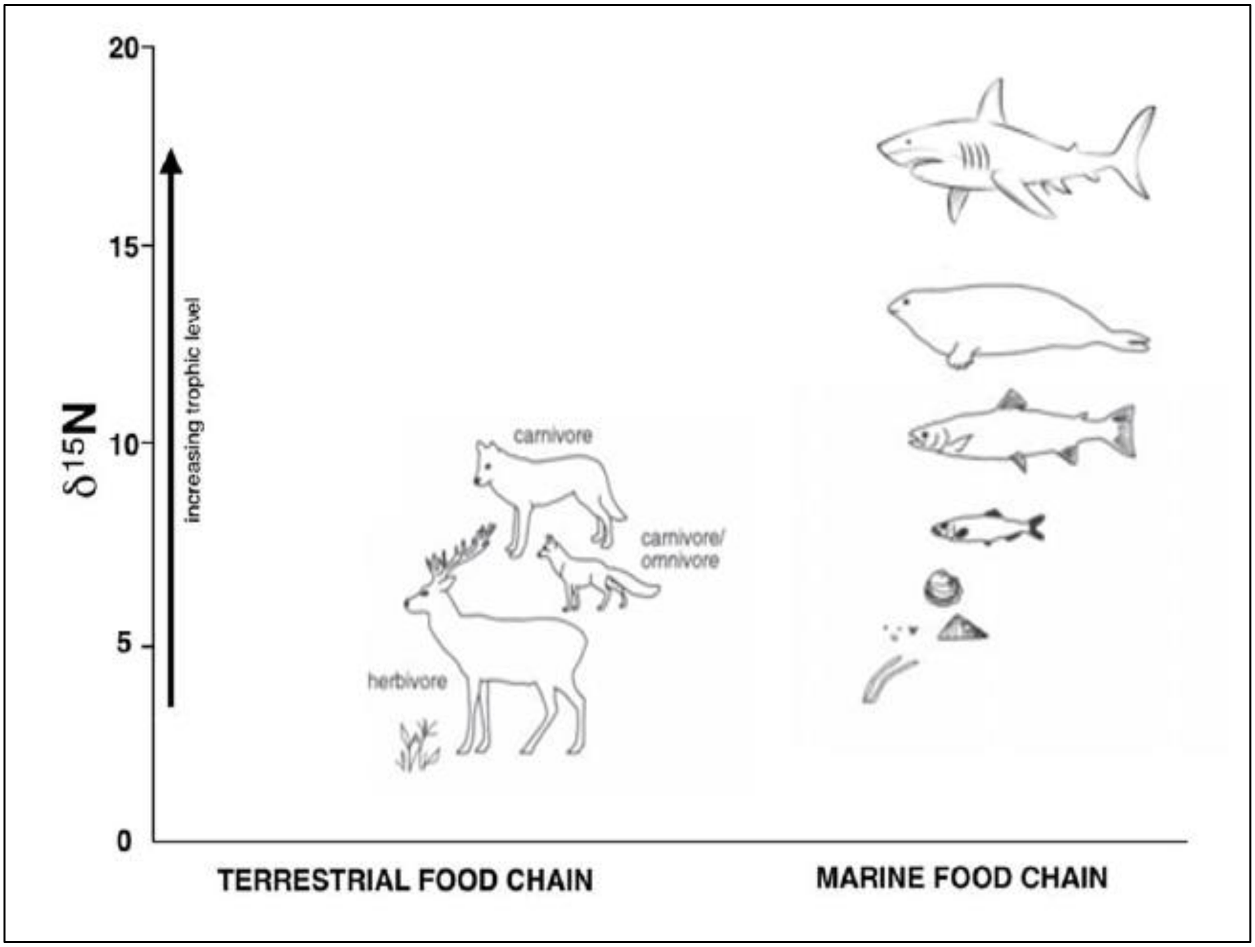


Figure 1: δ^{15}N stepwise enrichment pattern of both terrestrial and marine food webs (adapted from Schulting 1998: 205).

The degree of fractionation at each trophic level is usually considered to be about +2 to +4‰ (Ambrose 1991; Sponheimer *et al*., 2003a; Robbins *et al*., 2005; Hedges & Reynard 2007), although O'Connell *et al.* (2012) suggested that for humans, the value is closer to +6‰. The variation in ^{15}N in animals is influenced by a variety of factors including the δ^{15}N values of the nitrogen source, environmental stress, animal digestive physiology, and protein quality and quantity in the diet. Sponheimer *et al.* (2003b) found that the same species of herbivore showed larger diet-to-tissue (hair) fractionation on high-protein diets as compared with low-protein diets, with a difference of 2.3‰.

δ^{15}N values in marine food webs are generally higher than those on land, primarily because twice as much denitrification takes place in water compared with on land (Fowler *et al.,* 2013). In addition, terrestrial runoff containing isotopically enriched nitrogen compounds also contributes to the enrichment of marine food webs. There are more trophic levels in marine food webs, resulting in higher delta-^{15}N values in marine ecosystems; in comparison with terrestrial food webs and thus more opportunity for stepwise trophic fractionation (Schoeninger *et al.*, 1983; Schoeninger & DeNiro 1984; see Figure 1). For instance, a shark can be a fifth-order carnivore, a position that no terrestrial mammals can reach. For nitrogen-fixing organisms, δ^{15}N values may be close to 0‰, but they can reach as high as +20‰ for top consumers. Thus, a human that consumes a significant proportion of fish and/or marine mammals will have higher δ^{15}N values than one consuming purely terrestrial food.

In early studies, δ^{15}N values in consumers below about 10‰ were usually considered to indicate terrestrial diets, with higher values reflecting greater reliance on animal foods. δ^{15}N values above 10‰ were considered to indicate diets that included marine foods (Schoeninger *et al.,* 1983; Cox *et al.,* 2001). Further work has shown that this view is too simplistic. O'Connell (2023) notes that isotopic ratios are environmental signals rather than precise markers of specific food or water sources due to the variety and complexity of the numerous processes that result in isotopic fractionation. Nevertheless, in the Cape Town context, one would expect substantial intakes of animal and especially fish-based foods to lead to elevated consumer δ^{15}N values.

2.1.3. Stable Oxygen Isotopes

Oxygen is the most prevalent element on earth and has three stable isotopes ^{16}O, ^{17}O, and ^{18}O. ^{16}O and ^{18}O are the most abundant isotopes, contributing 99.757% and 0.205% respectively (Berglund & Wieser 2011). Most oxygen isotope studies to date have measured ^{18}O/^{16}O and reported the results in the delta (δ) notation relative to the international standards VSMOW (Vienna Standard Mean Ocean Water) and PDB.

Both organic and inorganic molecules, as well as molecular oxygen (O_2), are found in the biosphere. Global patterning in oxygen isotope ratios depends largely on the hydrological cycle (Luz *et al.,* 1984; Kohn *et al.,* 1996). δ^{18}O values in rainfall are affected by temperature, elevation, continental effect, reservoir effect (sources of water vapor), and amount of precipitation. Because less $H_2{}^{18}O$

evaporates at higher latitudes due to lower temperatures, there is less $\delta^{18}O$ in precipitation (Marshall *et al.,* 2007, McGuire & McDonnell, 2007).

The $\delta^{18}O$ of plant water is determined by the $\delta^{18}O$ of the water supply, the enrichment in ^{18}O that occurs during transpiration, and the exchange of water with organic molecules during the biosynthesis process (Barbour *et al*., 2005). When water is absorbed by plants or moved through their roots and stems, it is not isotopically fractionated. The leaves become enriched in ^{18}O when the $H_2{}^{16}O$ evaporates preferentially at the leaf (Marshall *et al*., 2007; Barbour *et al*., 2005). According to Helliker & Ehleringer (2002), elevated temperatures and reduced precipitation cause a rise in evaporation, which in turn causes leaves to enrich in ^{18}O.

The total $^{18}O/^{16}O$ of an animal's body fluids and tissues is determined by its drinking habits, food, body temperature, respiration, urination, bowel movement, and perspiration (Kohn *et al*., 1996; Lee-Thorp, 2008). According to Wong *et al*. (1988), wastewater that evaporates as vapor is deficient in ^{18}O, whereas wastewater that evaporates in liquid forms—such as urine and sweat—has an isotopic composition similar to that of body water. For unknown causes, the ^{18}O content of carnivorous species is often lower than that of herbivorous animals; on the other hand, proteins might be less enriched in ^{18}O than carbohydrates (Kohn *et al*., 1996).

Food and drinking water often follow the $\delta^{18}O$ values of adjacent ambient water, despite the fact that their oxygen isotope compositions vary broadly. Therefore, it is possible to trace the biological roots or migratory patterns of human beings using oxygen isotopes; however, it is unclear to what extent this tracing can be effective. Lightfoot & O'Connell (2016) showed that $\delta^{18}O$ values of humans from the United Kingdom and Europe were highly variable, to the extent that $\delta^{18}O$ is not helpful in establishing the area of origin. It should be noted, though, that their study encompassed mainly areas with similar $\delta^{18}O_{precipitation}$, excluding the Iberian Peninsula and other regions likely to show greater differences. Numerous factors, such as physiological processes and human behaviors (such food preparation techniques), can impact consumer $\delta^{18}O$: Royer *et al*., (2017) showed that cooking can produce shifts of up to 5.2‰ in $\delta^{18}O$.

Figure 2 shows the results of De Wet *et al.*'s (2020) investigation of the variations in $\delta^{2}H$ and $\delta^{18}O$ in tap water throughout South Africa. $\delta^{18}O$ values are very similar from Cape Town eastwards, but there are significant differences to the north, where the climate is much drier, and in parts of the interior of the country. δ^{18}O values might be effective in differentiating people from different parts of South Africa if these happen to have substantially different $\delta^{18}O_{water}$ values. It will not, however, be effective in all cases.

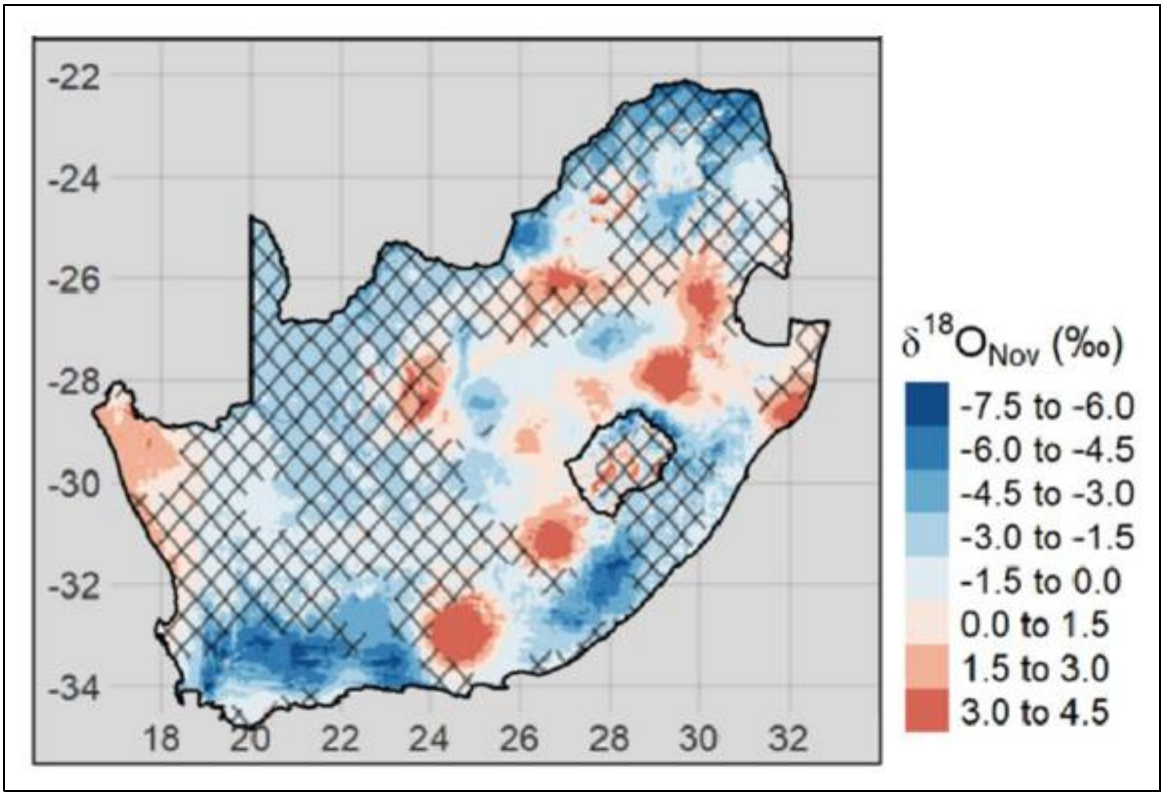


Figure 2: Modelled $\delta^{18}O_{SMOW}$ ranges for tap water in South Africa in the month of November 2017 based on universal kriging. Hatching indicates areas where the uncertainty of the model > 1 std dev of original data, from de Wet et al. (2020).

2.1.4. Strontium Isotopes

One of the best methods for determining the mobility of prehistoric humans and animals is to use strontium isotopes (Bentley 2006). There are four strontium isotopes that exist naturally: ^{84}Sr, ^{86}Sr, ^{87}Sr, and ^{88}Sr. Their respective natural abundances are 0.56%, 9.87%, 7.04%, and 82.53%. ^{87}Sr is a radiogenic isotope that is produced when ^{87}Rb decays (half-life approximately 4.9×10^{10} years), whereas ^{86}Sr is not radiogenic. ^{87}Sr/^{86}Sr is high in older geological formations and rocks that are rich in rubidium; in some parts of southern Africa, these have ^{87}Sr/^{86}Sr values as high as 0.8780 (House *et al.*, 2021; Wang *et al.*, 2023). Young volcanic rocks and marine systems have ^{87}Sr/^{86}Sr values around 0.703 and 0.709 respectively (Faure 1977, Sealy *et al.,* 1995, McArthur *et al.,* 2001, Sealy

2001, Müller & Anczkiewicz 2016). Because there is very little (~1%) mass difference between ^{87}Sr and ^{86}Sr, there is very little fractionation between the intake of strontium isotopes by plants and animals and the weathering of rocks. Thus, the ratio of ^{87}Sr to ^{86}Sr in living things is quite comparable to that of the underlying geology (Hurst & Davis 1981, Sillen *et al*., 1998, Montgomery *et al*., 2000). Any fractionation that occurs is normalized to the value of 0.1194 for ^{86}Sr/^{88}Sr so that the measured ^{87}Sr/^{86}Sr values will not be affected by fractionation (Knudson *et al*., 2010).

As strontium is absorbed by plants and found in soils, groundwater, and surface waters, it is a component of every ecosystem (see Figure 3 for details) (Bataille et al., 2020). ^{87}Sr/^{86}Sr in animals depends on the ratios of the soils (in Africa, usually derived from bedrock) over which the individual ranged and fed, together with inputs from dust, marine aerosols, or other sources. According to Brennan *et al*., (2016), ^{87}Sr/^{86}Sr in consumers can thus be utilized to reconstruct migration over several geographical locations with varying ^{87}Sr/^{86}Sr. Copeland et al. (2016) study utilized strontium isotopes to investigate ancient animal migration patterns in South Africa. By analyzing strontium ratios in tooth enamel, they were able to infer the geographic origins of individuals and track population movements over time. The application of strontium isotopes in anthropology can provide valuable insights into past human mobility and societal dynamics in prehistoric contexts.

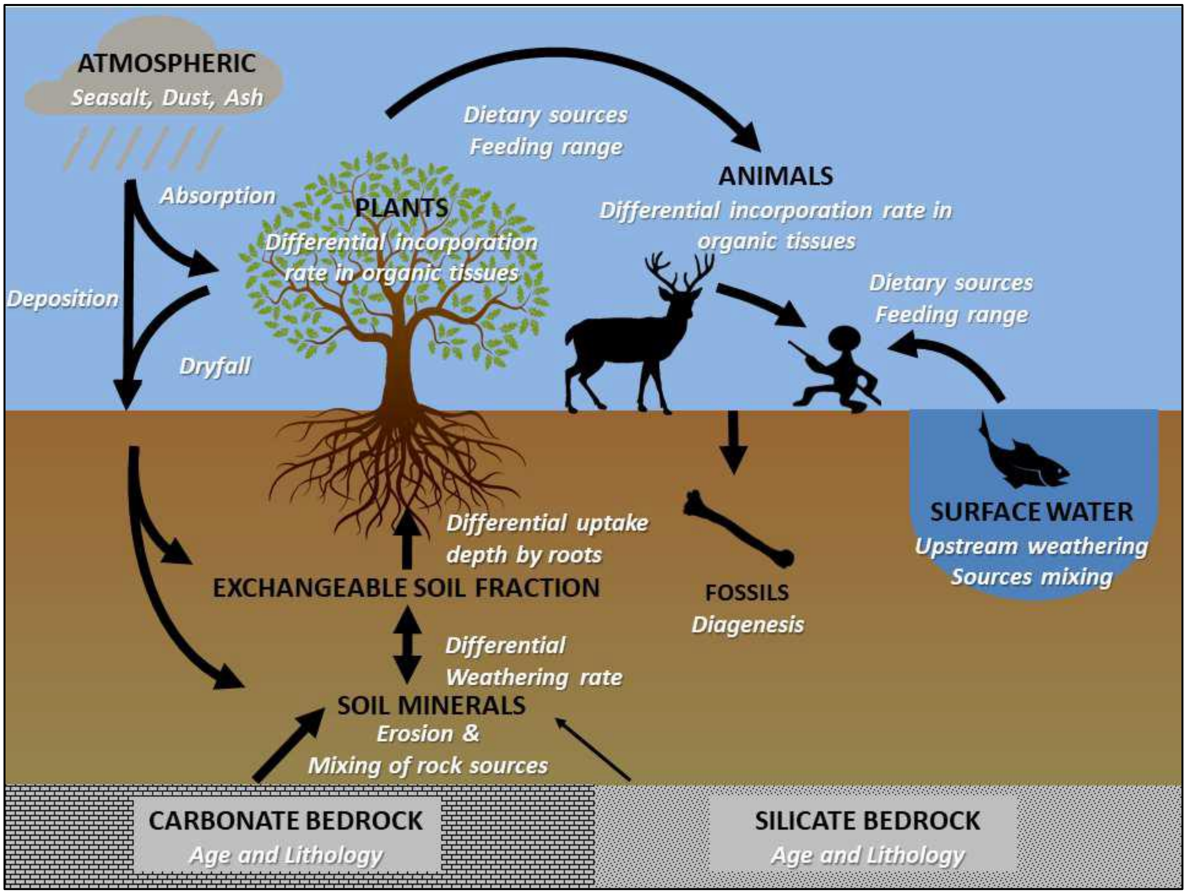


Figure 3: Strontium pathway (From Bataille *et al.,* 2020:7).

2.1.4.1. Geology of the Greater Cape Town Area.

The Malmesbury Group, Cape Granite Suite, and Table Mountain Group are the three primary hard-rock geological types found in the Greater Cape Town area. (See Fig. 4). In addition, substantial areas of near-coastal land surface are covered by geologically recent marine-derived sands (shown in yellow in Fig 4).

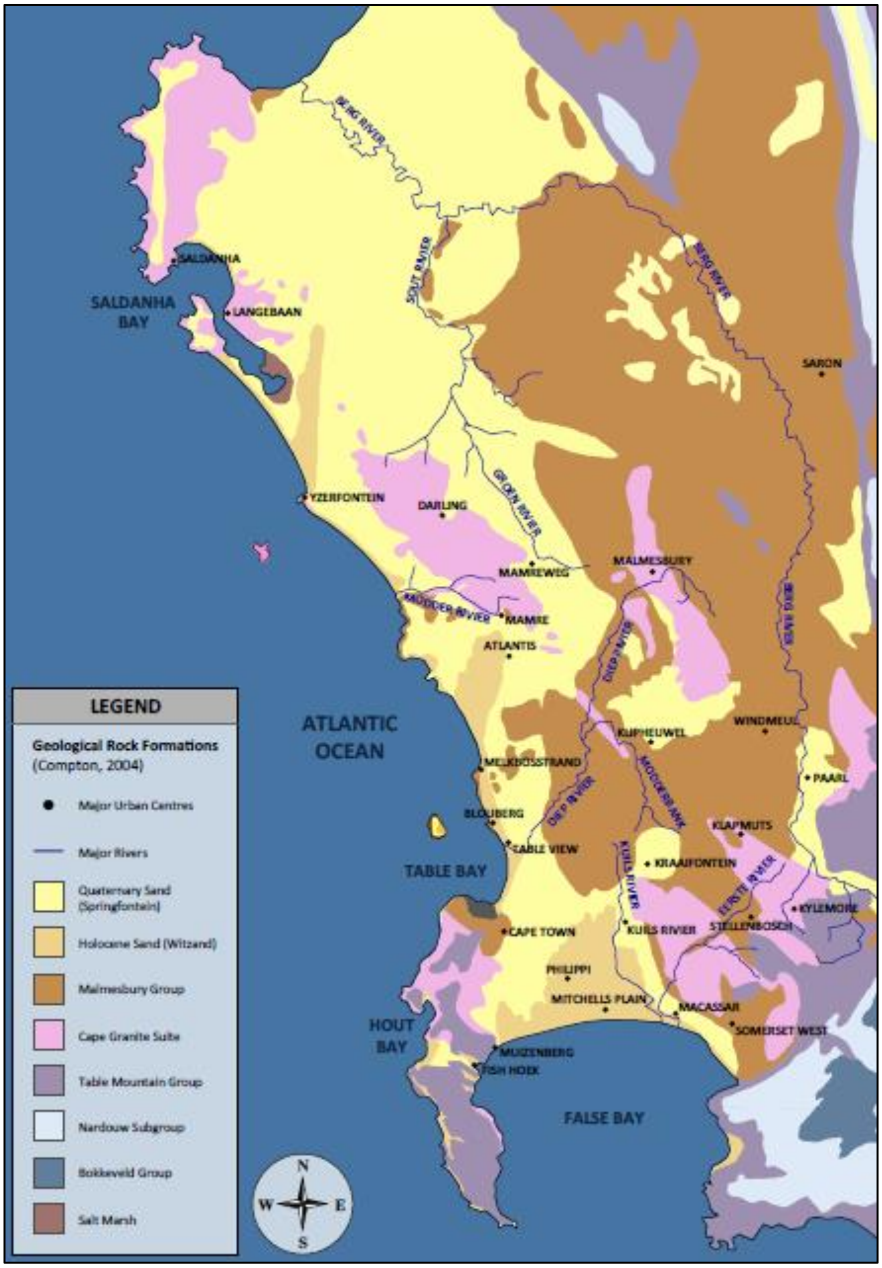


Figure 4: Geological map of the Cape Town area and surrounds (adapted from Compton 2004).

The oldest rocks in the Greater Cape Town region are the shales, greywackes, siltstones and hornfels belonging to the Malmesbury Group, which dates to 630 million years ago. These rocks were initially laid down as muddy sand and marine mud deposits. They are mostly dark in color which suggests inclusion of substantial amounts of organic material (Compton, 2004).

The Malmesbury Group Formation was repeatedly invaded by massive granite intrusions that make up the Cape Granite Suite, resulting in metamorphic processes that have created the indurated shale known as hornfels (Compton, 2004). On top of this base, about 450 million years ago, the Table Mountain Group's younger sedimentary strata were deposited (Compton, 2004). These are further divided into three formations, which are mostly made up of mudstones and sandstones, and are referred to as the Peninsula Formation, Pakhuis Formation, and Graafwater Formation. These later underwent lithification under pressure, and subsequent folding created the Cape Fold Belt Mountain chain (Compton, 2004).

The youngest geological substrates in the area are Quaternary limestones and Holocene coastal sands resulting from sea-level fluctuations during the late Cenozoic (Compton, 2004).

Throughout South Africa, strontium isotopes have been the subject of extensive research in various fields. The area most relevant to this thesis is the southwestern part of the country, especially the Cape Town area. Allsopp & Kolbe (1965) analyzed whole rock $^{87}Sr/^{86}Sr$ in Malmesbury shales and Cape granites and obtained values as high as 1.16 (See Table 1). Whole-rock values can, however, differ from bioavailable values, especially if different components of the rock have different $^{87}Sr/^{86}Sr$ and weather at different rates. Whole-rock values also do not reflect (as just one example) possible inputs of marine-derived strontium via sea mists, which are a common feature of the Cape Town area. To estimate bioavailable $^{87}Sr/^{86}Sr$, Sealy *et al*. (1991), Soderberg & Compton (2007), Radloff *et al.* (2010), Copeland *et al.,* (2016), Lehmann *et al.,* (2018) and Scott *et al.* (2020) all conducted studies in the Western Cape Province, analyzing mainly plants and animal tissues from various geological substrates. The $^{87}Sr/^{86}Sr$ values range from 0.7090 (for geologically recent marine-derived sands) to 0.7240 on the ancient substrates of the Cape Granite Suite and Cape Supergroup. In humans, we would therefore need to see values outside this range to be able to infer that the individual did not come from Cape Town.

Table 1: Previously published $^{87}Sr/^{86}Sr$ in biological and geological samples from southwestern South Africa (*Scott et al.,* 2020).

Author	**Sample Type & Location**	**Geological substrates of southwestern South Africa**								
		Malmesbury Shales	Cape Granite Suite	Cape Supergroup			Karoo Supergroup		Bredasdorp Group	Quaternary coastal sands
				Table Mountain	Bokkeveld	Witteberg	Little Karoo	Greater Karoo		
Allsopp & Kolbe, 1965	Rocks (Cape Peninsula)	0.7208-0.7873	0.7701-1.1602							
Sealy et al., 1991	Modern bone of mammals (Southwestern coast)	0.7178-0.7179		0.7154-0.7175						0.7094-0.7117
Soderberg & Compton, 2007	Plant and soil (Cape Floristic Region)			**Plants:** 0.722 0.724 **Soil:** 0.724						
Radloff et al., 2010	Modern teeth of rodents (De Hoop Nature Reserve)	0.7101-0.7104		0.7098-0.7100					0.7091-0.7099	**Dune strandveld:** 0.7092-0.7093
Copeland et al., 2016	Plants (Southern Cape)	0.7095-0.7157	0.7095-0.7177	0.7092-0.7169	0.7093-0.7209	0.7164-0.7237	0.7124-0.7202	0.7168-0.7237	0.7092-0.7101	
Lehmann et al., 2018	Modern bone and teeth and plants (Southwestern coast)	0.7141-0.7204	0.7114-0.7236	0.7141-0.7204						0.7094-0.7117
Scott et al., 2020	Animals and plants (Southwestern coast)		0.7115-0.7146	0.7099-0.7131			0.7152-0.7190		0.7091-0.7099	0.7093-0.7095

2.2. Tooth Structure and Composition.

The basic structure of all mammalian teeth, including incisors, canines, premolars, and molars, consists of the crown and the root. The innermost portion of the tooth is called the pulp chamber, which is home to blood vessels and nerves. The dentine makes up the second layer of the crown, while the enamel makes up the third and outermost layer. Cementum, a tissue that resembles bone, makes up the outermost layer of the roots (Ungar, 2010).

2.2.1. Enamel

Dental enamel is the tough and durable outer layer of tooth crowns, and it is known to be the strongest substance in the bodies of mammals, (Lacruz *et al.,* 2017). It is a composite material made to survive extraordinary chemical changes over the course of a lifetime in addition to withstanding the forces of chewing and shielding the tooth structure from outside injury (Gil-Bona & Bidlack 2020). The complex process of enamel formation and mineralization is controlled by cells known as ameloblasts (Thesleff 2003, Lacruz *et al*., 2017). The formation of enamel, or amelogenesis, occurs in two distinct stages: i) the laying down of an organic matrix and ii) mineralization (Ungar 2010). The organic matrix consists of protein, water, and minerals, initially each contributing approximately one-third. During mineralization, water and protein are resorbed and hydroxylated calcium phosphate or hydroxyapatite ($Ca_{10} [PO_4]_6[OH]_2$) crystallites are laid down (Boyde 1967). Over time, these become larger and increasingly dense, forming heavily mineralized mature enamel. Since enamel does not remodel, it might reveal details about a person's diet and geographic location while their crown is forming. The timing and duration of crown formation for different teeth is described below.

Mature enamel consists predominantly of calcium phosphate (96%), but includes substitutions involving ions such as fluoride, carbonate, and hydroxide, which can affect the properties of enamel, such as its hardness and resistance to decay. There is a very little organic matrix in mature enamel, only approximately 1%, consisting mainly of phosphoproteins and amelogenins (Boskey 1981). Large phosphate crystallites and little pore space characterize the very dense enamel. Because of this, enamel is less likely than dentine and bone to undergo diagenesis (Lee-Thorp & Sponheimer 2003).

2.2.2. Dentine

According to Ungar (2010), dentine is made up of about 70% minerals, 20% collagen and other proteins, and 10% water. Because dentine has less mineral than enamel, it is softer. Right after enamel mineralization begins, during tooth formation, primary dentine forms. In aged teeth, secondary dentine develops gradually and is typically correlated with a reduction in the quantity of functional cells. It shields the pulp from the elements. Consequently, fresh dentine grows as a localized response to the thinning enamel, forming the tertiary dentine that helps protect the tooth's surface as the enamel erodes (Ungar 2010). Dentine, like enamel, does not remodel (Gage et al., 1989); hence, information about an individual's food and residency during the era of tissue formation can be obtained from their isotopic composition. Collagen in tendons, skin, and bone is remarkably similar to that of dentine. It is synthesized largely from dietary amino acids, although where necessary, amino acids are formed from precursors available in the metabolic pool (Ungar 2010).

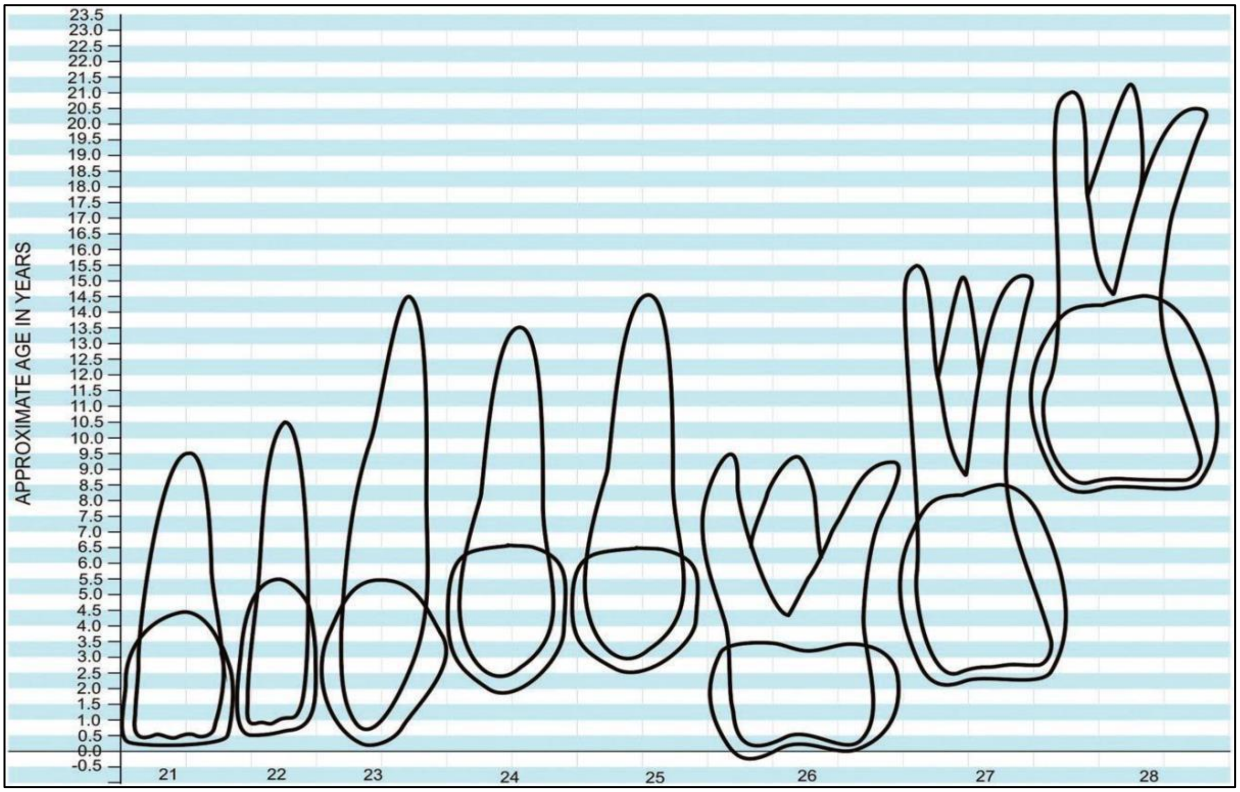
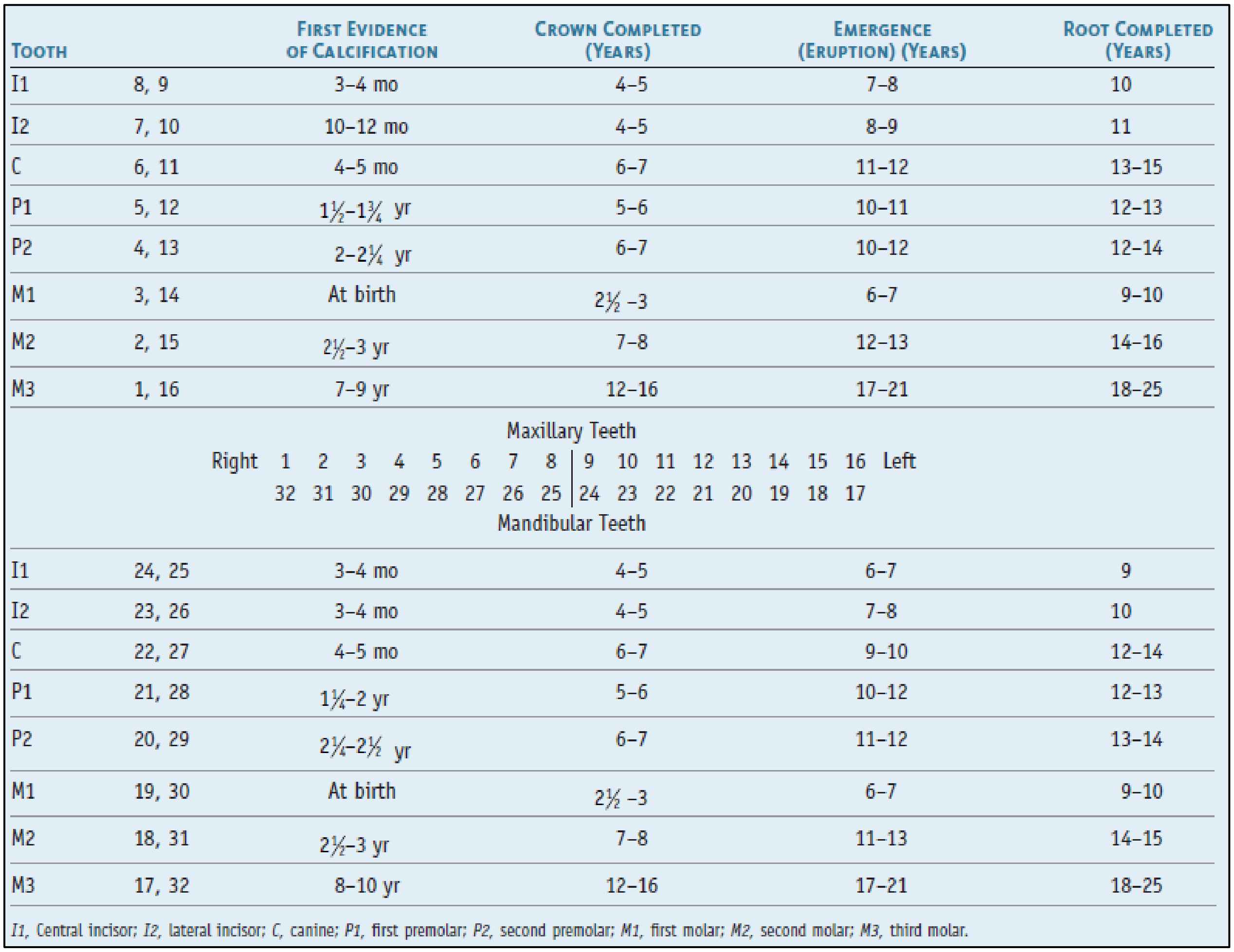


Figure 5: Ages of development of human permanent maxillary teeth (from left to right, first incisor to third molar) (from Beaumont & Montgomery, 2015: 409).

2.2.3. Age of tooth development

All teeth start to form from the occlusal surface. In humans, they develop at different stages between birth and the early 20s (Figure 5; Table 2) (Beaumont and Montgomery, 2015: 409). The earliest forming permanent teeth are the first molars with crown initiation (Ci) commencing at birth and crown completion (Cc) between 2.5-3 years. For both maxillary and mandibular first incisors and the mandibular second incisor, the Ci is approximately between 3-4 months and the Cc at approximately 4-5 years. For the maxillary first incisor, Ci is between 10-12 months and the Cc at 4-5 years. Maxillary and mandibular canines have Ci at 4-5 months and Cc at 6-7 years of age. The first maxillary premolars have Ci between 1.5-1.75 years and the mandibular first premolars have Ci between 1.25-2 years; both teeth have Cc approximately between 5-6 years. Maxillary second premolars have Ci between 2-2.25 years and mandibular ones between 2.25-2.5 years; both have Cc between 6-7 years. The second molars are the next teeth to develop, Ci for both the maxillary and mandibular second molars is between 2.5-3 years, with the Cc between 7- 8 years. The last teeth to erupt are the third molars. Ci takes place between 7-9 years for the maxillary M3 and for the mandibular M3 it happens between 8-10 years; the Cc for both occurs between 12-16 years (Nelson & Ash 2010). The interval between the development of the crown and the eruption of the tooth into the mouth is typically between 2-5 years, by this time, half to 2/3 of the root will have been created as well. Further root development happens after tooth eruption, and it takes 1.5-3.5 years on average for the root to completely form after tooth eruption (Nelson & Ash 2010).

Table 2: Chronology of permanent tooth development, eruption, and completion (From Nelson & Ash, 2010: 31).



TOOTH		FIRST EVIDENCE OF CALCIFICATION	CROWN COMPLETED (YEARS)	EMERGENCE (ERUPTION) (YEARS)	ROOT COMPLETED (YEARS)
I1	8, 9	3–4 mo	4–5	7–8	10
I2	7, 10	10–12 mo	4–5	8–9	11
C	6, 11	4–5 mo	6–7	11–12	13–15
P1	5, 12	1½–1¾ yr	5–6	10–11	12–13
P2	4, 13	2–2¼ yr	6–7	10–12	12–14
M1	3, 14	At birth	2½ -3	6–7	9–10
M2	2, 15	2½–3 yr	7–8	12–13	14–16
M3	1, 16	7–9 yr	12–16	17–21	18–25

Maxillary Teeth

Right	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Left
	32	31	30	29	28	27	26	25	24	23	22	21	20	19	18	17	

Mandibular Teeth

I1	24, 25	3–4 mo	4–5	6–7	9
I2	23, 26	3–4 mo	4–5	7–8	10
C	22, 27	4–5 mo	6–7	9–10	12–14
P1	21, 28	1¾–2 yr	5–6	10–12	12–13
P2	20, 29	2¼–2½ yr	6–7	11–12	13–14
M1	19, 30	At birth	2½ -3	6–7	9–10
M2	18, 31	2½–3 yr	7–8	11–13	14–15
M3	17, 32	8–10 yr	12–16	17–21	18–25

I1, Central incisor; *I2*, lateral incisor; *C*, canine; *P1*, first premolar; *P2*, second premolar; *M1*, first molar; *M2*, second molar; *M3*, third molar.

2.3. Bone Collagen

The main proteinaceous substance found in bone is collagen, which gives the bone its flexibility and makes up more than 90% of the organic matrix (Weiner & Wagner 1998; Taton 2001). About 33% of collagen is made up of glycine, and the remaining 20 to 25% is made up of proline and hydroxyproline together (Schwarcz & Schoeninger, 1991). Throughout the process of bone formation and subsequent maturation, apatite crystals with a length of 10 to 50 nanometers are inserted into the gaps created by the collagen fibril matrix (Taton 2001) (See Figure 6).

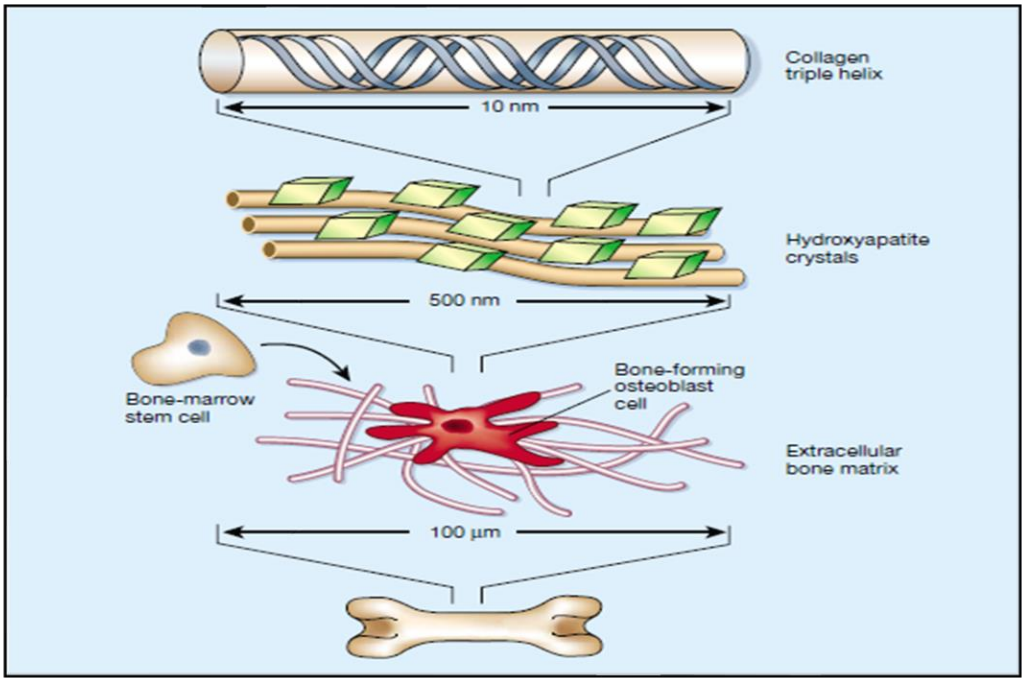


Figure 6: The structure of bone at different scales (From Taton 2001: 491).

Although the collagen fibrils are first created during bone development, they resorb and re-form (re-model or "turn over") over the course of a person's lifetime. Therefore, the isotope values for bone collagen reflect the diet at the time the collagen formed and or remodeled. Although biological, physical characteristics and structure of the skeletal system influence the rate of turnover, it is slow enough that some skeleton parts reflect an individual's dietary patterns over a long period of time and the environment they lived in for most if not all of their life (Parfitt, 2002; Lee-Thorp, 2008). After 50 years of age, the cortical bone of the sixth rib has a typical turnover rate of 4% every year. In healthy postmenopausal women, the mean turnover in iliac cortical bone is 7.7%/year and 17.7%/year in the iliac cancellous bone (Parfitt 2002). Since the collagen in the human femoral mid-shaft bone ages more slowly, it takes longer to reflect a person's diet. Hedges et al. (2007) have shown that even in individuals 50 years of age or beyond, the femoral mid-shaft retains some of the collagen that was deposited during adolescence.

2.4. Stable isotope studies on human skeletal remains.

When stable isotope analysis focusing on human skeletal remains commenced, it was employed to investigate a small range of questions, e.g., the advance and spread of maize agriculture (van der Merwe & Vogel 1978), or marine food consumption in the Mesolithic and Neolithic in northwestern Europe (Tauber 1981, Schulting & Richards 2001, Schulting *et al.,* 2003). The questions addressed by stable isotopic analysis are now much more diverse, with themes related to diet, subsistence, human movement, origins, and social practices of humans (Lee-Thorp, 2008, Makarewicz & Sealy, 2015).

Examining people's origins and dietary patterns, particularly how these may have changed over time, is a significant issue in recent studies that is most pertinent to this thesis. The stable isotope ratios of carbon, nitrogen, and oxygen along with strontium can be used to follow the migratory patterns of individuals or animals from a location (or diet) with one isotopic "signature" to another that is notably different. (Meier-Augenstein & Fraser, 2008; Schroeder et al., 2009; Kamenov *et al*., 2014; Price *et al*., 2020; Fauberteau *et al*., 2021).

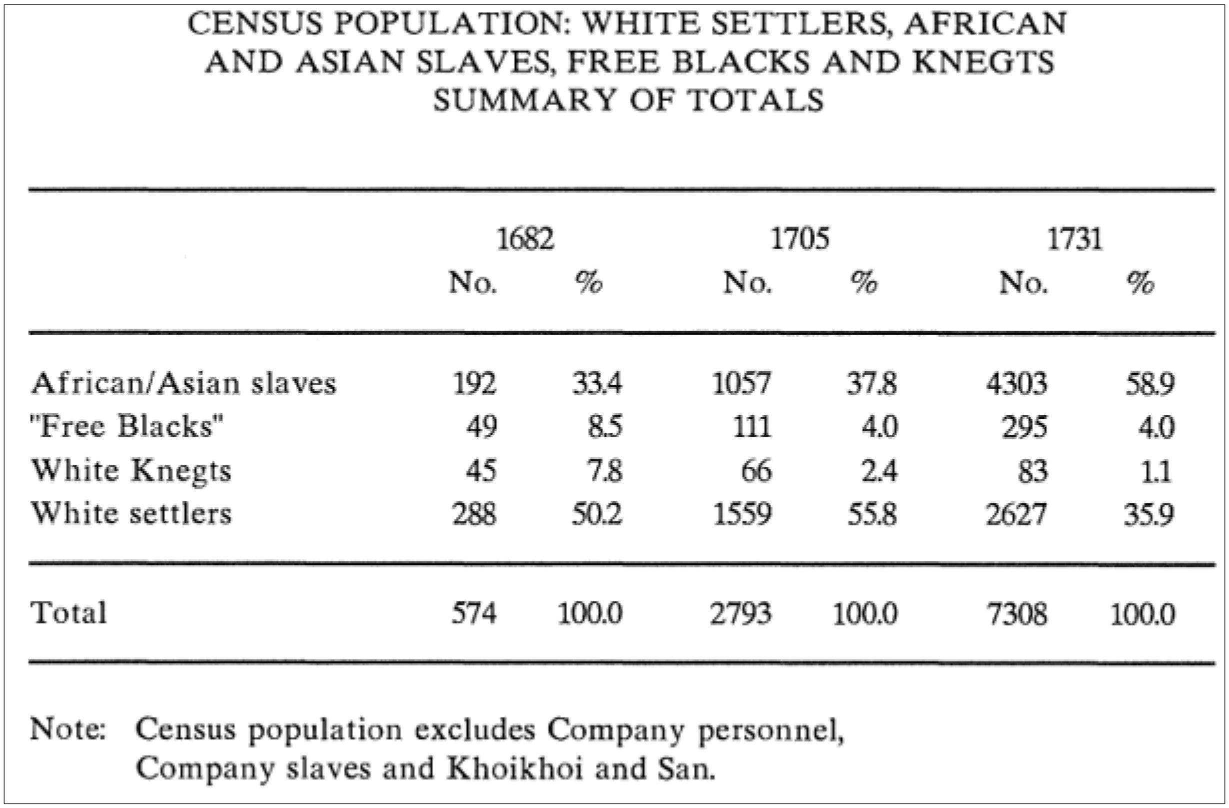
Many studies compare different body tissues, including hair, nails, bone, and teeth, that offer isotopic information on various phases of a person's life history, because of their distinct histories of formation and turnover (Bartelink & Chesson 2019). As outlined above, teeth do not remodel after they are formed; but, if a tooth is broken later in life, secondary reparative dentine may be added. As a result, teeth capture the isotopic structure of a person's diet and region of origin. The majority of skeletal components continue to change throughout life, with some forming throughout childhood and adolescence. Therefore, a long-term average of diet can be obtained through analysis of bone collagen. There are no consistent offsets between δ^{13}C and δ^{15}N in dentine and bone collagen, according to studies reporting these values (France & Owsley 2013; Salazar-Garcia *et al.,* 2014; van der Merwe *et al.,* 2003). Tissues such as nails and hair grow more quickly and do not remodel. A strand of hair, for instance, provides information about a person's food and surroundings over the course of several months because hair grows at a rate of approximately 1 cm each month and continues to grow throughout life (Meier-Augenstein & Kemp 2012; Ehleringer *et al.,* 2020; Bataille *et al.,* 2020). Comparison of the isotopic composition of different body tissues can therefore provide a powerful tool for tracing changes in someone’s diet and place of residence over their lifetime.

2.5. Stable isotope studies of pre-colonial and historical skeletons in the Greater Cape Town area.

There have been a number of papers using stable isotopes as palaeodietary tracers in Holocene hunting and gathering communities of the Western Cape coast (summarized in Sealy 2016), and also amongst pre-colonial sheep and cattle herders of the last 2000 years (Sealy, 2010). This is a winter-rainfall area, with winter spanning between June and September; so terrestrial vegetation is mostly C_3. If we consider just the Cape Peninsula and immediately adjacent areas, $\delta^{13}C_{\text{bone collagen}}$ values for 35 hunter/foragers who lived between 7000-2000 years ago vary from -17.9 to -10.6‰ and $\delta^{15}N_{\text{bone collagen}}$ values from 10.2 to 17.3‰ (Lewis & Sealy 2018). Lower values result from the consumption of diets based on terrestrial foods, while the most positive values are for specialized marine foragers who consumed diets composed very largely of seafood. The isotope measurements from these studies have recently been compiled in the AfriArch isotopic database (https://openarchaeologydata.metajnl.com).

The Dutch East India Company settled at Table Bay in the middle of the 17th century in order to supply ships that were sailing between Europe or the Americas and the east coast of Africa and Asia. It grew into an international port that is today the city of Cape Town. There was a large immigrant population including an eclectic mix of people from different continents, some of whom came voluntarily, while some were brought as slaves. Throughout the 17th and 19th centuries, the population of Cape Town consisted of the native Khoi people, workers of the Dutch East India Company (VOC), free burghers, slaves, free Black people, and *bandieten* (offenders sentenced to hard labor). According to the census of 1731, the population of the Cape consisted of 4303 African/Asian slaves; 295 ‘Free Blacks’; 83 White Knegts, and 2627 White settlers, in addition to the employees and slaves of the Dutch East India Company (See Table 3).

Table 3: Population diversity in the Cape during the 18th century (From Guelke 1988: 459).



CENSUS POPULATION: WHITE SETTLERS, AFRICAN AND ASIAN SLAVES, FREE BLACKS AND KNEGTS SUMMARY OF TOTALS

	1682		1705		1731	
	No.	%	No.	%	No.	%
African/Asian slaves	192	33.4	1057	37.8	4303	58.9
"Free Blacks"	49	8.5	111	4.0	295	4.0
White Knegts	45	7.8	66	2.4	83	1.1
White settlers	288	50.2	1559	55.8	2627	35.9
Total	574	100.0	2793	100.0	7308	100.0

Note: Census population excludes Company personnel, Company slaves and Khoikhoi and San.

The diverse population and associated historical and archaeological questions led to the first multi-isotope study of the geographical origins of humans. Sealy *et al.*, (1993, 1995) measured ^{87}Sr/^{86}Sr, δ^{13}C and δ^{15}N in the teeth, rib and femoral bone of a female skeleton excavated from the presumed slave lodge on the historic farm Vergelegen, Somerset West. There were large isotopic differences between her childhood diet, as reflected in her teeth (dentine), and a more mixed adult diet, reflected in the bone collagen of her rib and femur. δ^{13}C values varied from -13,2‰ to -10,9‰ and δ^{15}N from 6.7 to 12.4‰. In addition, values for enamel ^{87}Sr/^{86}Sr were higher than any recorded in the Western Cape. This major change in diet and place of residence was almost certainly the result of her capture as a slave, probably in sub-tropical Africa, and transport to the Cape.

Subsequent development of the multi-isotopic, multi-skeletal element approach has enriched our understanding of the Cape during the 17th-19th centuries. Cox & Sealy (1997) examined the diets and origins of the slaves on board the Portuguese ship, Pacquet Real, when she was wrecked in Table

Bay in 1818. An extended series of analytical studies of skeletons excavated from colonial burial grounds in Cape Town enabled the identification of first-generation slaves from both Africa and Indonesia, and the correlation of different grave styles with regions of origin (Cox *et al.,* 2001).

More recent work has suggested that male immigrants had more varied geographical origins than females (Kootker *et al.,* 2016; Mbeki *et al.,* 2017). These studies are remarkable for the very large ranges of $^{87}Sr/^{86}Sr$ and $\delta^{13}C$ and $\delta^{15}N$, values reported, in part because of the mix of immigrants and locally born people. At the 18th-19th century site of Cobern Street, $^{87}Sr/^{86}Sr$ values ranged from 0.70600 to 0.73605; $\delta^{13}C_{dentine}$ values from -19.80‰ to -5.34‰ and $\delta^{13}C_{cancellous}$ values from -18.80‰ to -8.60‰ (Kootker *et al.,* 2016). Working with a different group of 18th-19th century skeletons, the study also reported large ranges of $\delta^{13}C$ and $\delta^{15}N$; $\delta^{13}C_{dentine}$ values ranged from -20.3‰ to -6.6‰ and $\delta^{13}C_{cancellous}$ values from -21.1‰ to -8.0‰; $\delta^{15}N_{dentine}$ ranged from 7.1‰ to 16.3‰. and $\delta^{15}N_{cancellous}$ from 9.8‰ to 16.5‰ (Mbeki *et al*., 2017). Some of the variability here is due to some of these people being immigrants, but it is, however, clear that in this region, locally available foods can lead to very diverse $\delta^{13}C$ and $\delta^{15}N$ values.

2.6. Interpreting isotopic variation

When interpreting isotope measurements, a challenge arises due to uncertainty about the extent to which intra- and inter-individual variation can be attributed to physiological or metabolic differences. It is difficult to decide what thresholds should be used for interpreting differences as behavioral rather than simply biological.

DeNiro & Schoeninger (1983) found variations of up to 1‰ in $\delta^{13}C_{collagen}$ and 1.4‰ in $\delta^{15}N_{collagen}$ values of collagen from 15 mink consuming isotopically monotonous diets.

Olsen *et al*. (2014) investigated intra-individual variation in historical Western European skeletons. They found within-individual differences no greater than 0.4‰ for $\delta^{13}C_{collagen}$ and 0.9‰ for $\delta^{15}N_{collagen}$ values, although the sample size was small (n=6-8). Fahy *et al*. (2017) analyzed ten early medieval skeletons from Canterbury, England. They reported a maximum intra-individual difference in $\delta^{13}C_{collagen}$ of 1.6‰, while for $\delta^{15}N_{collagen}$ it was 3.1‰. In a geographically wide-ranging study, Berg *et al*. (2022) compared $\delta^{13}C$ and $\delta^{15}N$ in five to six long bones from each of 27 modern individuals from the USA, Philippines, Korean Peninsula and Vietnam/Laos. Within-individual $\delta^{13}C_{collagen}$ and $\delta^{15}N_{collagen}$ varied by up to 0.78‰ and 1.12‰ respectively, while $\delta^{13}C_{bioapatite}$ varied by up to 1.63‰ and $\delta^{18}O_{bioapatite}$ by up to 4.80‰. These authors propose that two bones with $\delta^{13}C_{collagen}$ values that differ by more than 0.75‰ are most likely from different individuals, and those with differences greater than 0.95‰ are certainly from different individuals. Similarly, they argue that differences in $\delta^{15}N_{collagen}$ values bigger than 1.05‰ possibly indicate different individuals and those larger than 1.35‰ definitely do. These cut-off points are lower than those recommended by Plomp *et al*. (2020), who argue that intra-individual differences greater than 2‰ for $\delta^{18}O$ and $\delta^{13}C$ are crucial in demonstrating changes in diet or geographic location.

Based on the current literature, therefore, a conservative approach would be to interpret only intra-skeletal differences of greater than 2‰ in $\delta^{13}C$ as resulting from behavioral (dietary) change. The threshold for $\delta^{15}N$ may be higher, based on Fahy *et al*. (2017).

CHAPTER THREE: Materials and Methods

3.1. Introduction

The study sampled 37 skeletons from Cape Town and nearby areas. These remains are curated in the Human Skeletal Repository of the Department of Human Biology at the University of Cape Town Medical School. These individuals are forensic cold cases (listed as "forensic" in the Accessions Register of the Human Skeletal Repository) that were recovered in the Cape Town Greater Cape Town Area. Their residential histories and identities are unknown. This chapter outlines what samples were taken, and how they were processed. Laboratory procedures followed in this study will be described, beginning with the preparation of the samples, and finally the isotopic analyses.

3.2. Sampling strategy

Samples were acquired from the Human Skeletal Repository at the UCT Medical School, with a permit obtained from the University's Human Remains Ethics Committee (*HREC REF: 333/2021).* For this study, no juvenile individuals were selected; the focus was on adults aged 18 and older.

Individuals were selected for sampling based primarily on geography: all came from Cape Town or nearby (with the furthest coming from Montagu, a small town approximately 186 km east of Cape Town). The second criterion had to do with which skeletal elements were present. Many skeletons in the Human Skeletal Repository are incomplete, and even complete individuals may be edentulous. The aim was to sample a rib bone and a tooth (preferably an early-forming tooth) per individual to compare the isotopic values from the person's early life (as reflected in the tooth) with later life (reflected in the rib). Preference was given to complete skeletons that had not been altered, for instance, by burning. Initially, skeletons were selected only if both ribs and teeth were preserved, preferably incisors or first molars. Subsequently, some additional individuals were included even if they did not have teeth, to increase the sample size. In some cases, teeth other than incisors or first molars were sampled if the preferred teeth were missing or so firmly fixed in their sockets that they could not be removed without damage. The teeth selected were easy to remove or already out of their sockets. When sampling ribs, rib fragments were preferred if available but if not, a piece of rib approximately 1cm long was cut from a damaged or incomplete rib if present, avoiding any pathological conditions.

3.3. Tooth Enamel

3.3.1. ^{87}Sr/^{86}Sr

The first analysis to be carried out was the measurement of ^{87}Sr/^{86}Sr in tooth enamel by *in situ* laser ablation, because it was the least destructive methods. Analyses were done in the Department of Geological Sciences at the University of Cape Town using the Nu Instruments laser ablation multi-collector inductively coupled plasma mass spectrometer (LA-MC-ICP-MS) coupled to an Australian Scientific Instruments RESOlution SE laser. This study followed the basic procedures reported in le Roux *et al.,* (2014), using a laser spot size of 80 μm operating at 30 Hz and 1.15 kV yielding an energy density of 0.8 J.cm^{-2}. Ablation was done using helium, set at 400 ml.min^{-1}, and nitrogen, set at 0.9 ml.min^{-1}, as a sweep gas mixture in the sample chamber and mixed later with argon, set at 0.95 L.min^{-1}, prior to injection into the plasma. The whole tooth was placed in the sample chamber (See Figure 7) and the analysis line (400 μm long) was cleaned before ablation by sweeping the laser along the analysis path using a 100 μm laser spot. The path was selected carefully to avoid any cracks or defects in the enamel. Horizontal measurements were taken on the occlusal, middle, and cervical parts of each tooth. A modern shark tooth analyzed repeatedly throughout the data acquisition session as the in-house standard yielded ^{87}Sr/^{86}Sr results (0.70905 ±0.00016; n = 11) in agreement with the ^{87}Sr/^{86}Sr value for modern seawater of 0.709175 (McArthur *et al.,* 2001; Müller & Anczkiewicz 2016).

Figure 7: Teeth ready for analysis in the sample chamber of the LA-MC-ICP-MS.

3.3.2. δ^{13}C and δ^{18}O

3.3.2.1. Sampling

Samples of tooth enamel for measurement of δ^{13}C and δ^{18}O were taken from the lingual (tongue) or buccal (cheek) surfaces. The surface of the area to be tested was softly abraded to eliminate any superficial contamination using a portable Dremel rotary drill equipped with a 0.5mm diameter diamond-tipped drill bit before the enamel powder was collected. In order to determine the average diet of the person during the period of crown creation, a sample was collected along the whole height of the tooth crown, from the occlusal surface to the cementum-enamel junction. Enamel was carefully removed, leaving the underlying dentine intact. After being gathered on a sanitized piece of lab weighing paper, enamel powder was put into 2ml microcentrifuge tubes. Approximately 4-6mg of enamel was obtained from each tooth to make sure that at least 2mg of processed enamel would be available for analysis.

3.3.2.2. Enamel pre-treatment

With certain adjustments, the enamel was prepared using the procedures described by Lee-Thorp *et al*. (1997). To remove any organic compounds, 1ml of ~1,75% v/v sodium hypochlorite (NaOCl) was applied to the enamel powder and left in for 45 minutes. Following a one-minute centrifugation, the samples were washed three times with distilled water. To eliminate any soluble mineral components, each sample was subsequently treated for 15 minutes with 0.1M acetic acid (CH_3COOH) (Loftus & Sealy 2012). Among them were absorbed carbonates, which might be effectively eliminated in this way because they are more soluble than structural carbonates (Webb *et al*., 2014). After centrifuging and rinsing the enamel samples three times with distilled water, the samples were freeze-dried.

3.3.2.3. Measurement of $\delta^{13}C$ and $\delta^{18}O$

For each dry, pre-treated enamel sample, 1.9 to 2.1mg of material was carefully weighed into a 12ml borosilicate glass tube that had been cleaned using phosphoric acid, following the method outlined by Loftus & Sealy (2012). The tubes were sealed using screw-top lids featuring septa and positioned within a temperature-controlled sampling tray set to 72°C in a Thermo Finnigan Model II gas bench. The CTC Analytics A200S autosampler was used to flush the tubes with helium. After manually adding five to seven drops (depending on the size of the sample) of 100% phosphoric acid (H3PO4) through the septum of each sample tube with a syringe, the carbonate in the bioapatite was allowed to react with the acid for at least two and a half hours, releasing carbon dioxide (CO_2). The gas that evolved in each tube was sampled by the autosampler. Finally, using a Delta Plus XP isotope ratio mass spectrometer (IRMS) managed by Isodat software, the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios in the pure CO_2 were determined. The standard deviations of repeated measurements of the standards Cavendish Marble and Carrara Z New were 0.2 for both $\delta^{13}C$ and $\delta^{18}O$. The outcomes are shown in relation to the Vienna PDB using the delta notation (δ).

3.4. Dentine and Bone Collagen

3.4.1. Sampling

The same process was followed in the extraction and pre-treatment of dentine and bone collagen. Small samples of dentine were obtained from the apices of the tooth roots (See Figure 8). Using a Dremel tool fitted with an emery disc (Dremel cut-off wheel N$^{o.}$ 409), the surfaces of the parts of the tooth roots to be sampled were lightly abraded to remove superficial contamination and any adhering cementum. This emery disc was changed after every sample to avoid contamination. Approximately 3-5mm of the root tip was then sampled to provide the 0.4-0.45mg of collagen required for analysis. For ribs, approximately 1 cm long fragments of the ribs were surface cleaned in the same way as the tooth roots, then cut off using the emery disc. A mask was worn throughout the extraction process and a new set of gloves was worn to handle each sample.

Figure 8: Photograph showing tooth after root tip was removed for analysis (Picture by Chakanetsa).

3.4.2. Collagen Extraction

The methods followed in this study are the standard procedures used in the Stable Light Isotope Laboratory at UCT. Each sample was placed in a 12ml pre-weighed round-bottomed glass tube with a screw cap. The tubes were then weighed again, to record the original weight of the bone or dentine for later determination of the collagen yield. Approximately 10ml of a defatting solution consisting of water, methanol, and chloroform (2.0:1.0:0.8 v: v) was added to each tube. The tubes were then placed on a Stuart rotator (SB3) and left to spin at room temperature for at least 24 hours. The defatting solution was then poured off and replaced with new solution, and this step was carried out twice. The samples were then rinsed three times in distilled water before being placed in 25 ml glass beakers (pre-cleaned with sodium hypochlorite (NaOCl), three rinses with distilled water, then oven-dried) and left in the fume hood for 48hrs to dry before being re-weighed to determine if the weights had changed from those measured originally.

The samples were then soaked in 0.2M hydrochloric acid (HCl) at room temperature to remove the mineral fraction. The acid was replaced after every 24 hours for up to 4 days until the samples were soft. Rib bones became somewhat translucent and flexible "pseudomorphs" of the original bone fragments, while dentine softened but did not become translucent. Softening was tested by probing with a sewing needle. In a few samples, small linear structures (blood vessels or plant roots) became visible as decalcification progressed. These were stripped or scraped away. To get rid of any base-soluble impurities such as humic acid, the decalcified samples were immersed in 0.1M sodium hydroxide (NaOH) for eighteen hours after being rinsed three times in distilled water (Ambrose 1990). After that, the samples were rinsed three more times in distilled water.

The next step was to gelatinize the samples in 10ml pH 3 water (acidified with HCl) at 75°C. Sample tubes were placed in a metal block on a laboratory hot plate in a fume hood, and the pH 3 solution was topped up every 48 hours. A marble was put on top of each tube to reduce evaporation and all the tubes in the block were loosely covered with aluminum foil. Once each sample was fully gelatinized, it was removed from the block heater, covered with parafilm, and put in the freezer. After all the samples had been gelatinized and frozen, they were taken out of the freezer. Holes were poked in the parafilm using a needle and the samples were freeze-dried.

3.4.3. Measurement of $\delta^{13}C$ and $\delta^{15}N$

For each sample, 0.40 - 0.45mg of extracted collagen was weighed into a tin capsule on a Sartorius MSE3.6P-000-DM microbalance. Weights were determined to the precision of one microgram. After that, the capsules were folded to contain the sample and keep air out. In order to produce CO_2 and N_2 gases, all samples were burned in a Flash 2000 organic elemental analyzer that was set to 1020°C. Helium was used as the carrier gas in the purification process of the gases before they were fed into a Delta V Plus isotope ratio mass spectrometer (IRMS) through a Conflo IV gas management unit. Germany's Thermo Scientific, located in Bremen, makes all three. Along with the samples, internal standards such as Choc, Sucrose, New Merck Gel, and Valerie were weighed and examined. For every run, seven aliquots of every standard were measured. Our lab or others have calibrated the internal standards against IAEA (International Atomic Energy Agency) standards. The standard deviations of repeated measurements of the standards were 0.2 for $\delta^{13}C$ and 0.1 for $\delta^{15}N$. $\delta^{15}N$ values are expressed relative to atmospheric nitrogen, and $\delta^{13}C$ relative to Vienna PDB.

CHAPTER FOUR: RESULTS

4.1. Introduction

This chapter presents the results of the measurement of $^{87}Sr/^{86}Sr$, $\delta^{13}C$, and $\delta^{18}O$ in tooth enamel, and $\delta^{13}C$ and $\delta^{15}N$ in bone collagen and dentine collagen. The list of all the individuals that were selected for this study, the skeletal elements sampled, and the $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $^{87}Sr/^{86}Sr$ values of dentine collagen, bone collagen (with collagen quality indicators), and enamel are presented in Table 4 below.

The quality of the extracted collagen was assessed from the collagen yield, weight % C, weight % N, and atomic C: N ratios (See Table 4 below).

Weight % C in these samples ranges from 26.8 to 43.4% (excluding the two samples highlighted in yellow: UCT 340/24402 (rib) and UCT 407/24384 (dentine), at 68.7 and 52.4% C respectively), and weight % N from 9.7 to 17.5% (excluding UCT 340/24402, rib with only 5.7% N). Atomic C:N values range from 3.1 to 3.3 except for UCT 340/24402 rib and UCT 407/24384 dentine, which have C:N of 14 and 3.5 respectively. All other C:N values fall within the ranges previously stipulated by Ambrose (1990) and Van Klinken, (1999), of 2.9 to 3.6 for ancient collagen and (allowing for rounding off) the range of 3.0 to 3.28 proposed for modern collagen by Guiry & Szpak (2020).

In UCT 340/24402 %N is low (5.7) and %C high (68.7), yielding C:N of 14.0. For UCT 407/24384, %N and %C are both high (17.5 and 52.4 respectively), leading to C:N of 3.5. These two samples may have been contaminated with humic matter, leading to high %C, or there may still have been some lipids present. These two samples are excluded from the further analysis of the results below.

Table 4: All isotope results and collagen quality indicators. One bone collagen sample (UCT 339/24530) was taken from the right radius shaft rather than a rib. Values highlighted in yellow fall outside the expected range for modern collagen. In the $^{87}Sr/^{86}Sr$ columns, O stands for occlusal, M for middle and DEJ for the Dentine Enamel Junction

UCT Acc. No.	Tooth	Dentine Collagen						Rib Collagen						Dentine & Bone		Enamel						
		$\delta^{15}N$	$\delta^{13}C$	Wt. % N	Wt. % C	Atomic C:N	Collagen Yield (%)	$\delta^{15}N$	$\delta^{13}C$	Wt. % N	Wt. % C	Atomic C:N	Collagen Yield (%)	Offset $\delta^{15}N$	Offset $\delta^{13}C$	$\delta^{13}C$	$\delta^{18}O$	$^{87}Sr/^{86}Sr$				
																		O	M	DEJ	Mean	Range
105	RI^1	15	-12,3	14,5	40,5	3,3	0,47	13,7	-12,6	11,9	32,7	3,2	0,73	1,3	0,3	-10,7	-1,5	0,7099	0,7099	0,71	0,7099	0,0001
136	LI_1	10,4	-8,7	11,1	31,4	3,3	2,72	11,2	-11	11,8	32,8	3,3	1,33	0,8	2,3	-1,3	-0,3	0,7188	0,719	0,7187	0,7188	0,0002
155	LI_1	11,7	-17,2	10,5	28,8	3,2	0,47	12,2	-15,8	12,3	33,3	3,1	5,78	0,5	1,4	-11,2	-5	-	-	-	-	-
233	RI_1	13,3	-11,3	12,7	35,1	3,2	4,82	13,3	-14,2	12,9	34,6	3,1	1,66	0	2,9	-5,6	0,8	0,7162	0,7159	0,7161	0,7161	0,0003
339								14,8	-15,8	13	35,2	3,2	3,28									
340	$LI_{1/2}$	12,9	-15,3	11,3	31	3,2	0,65	15,4	-18,5	5,7	68,7	14	1,71			-6,4	-2,8	0,7132	0,7133	0,7129	0,7131	0,0004
352	RC^1	14,6	-14	11,5	31,4	3,2	0,69	13,1	-13,9	10,2	27,9	3,2	7,02	1,5	0,1	-7,2	-2,9	0,7128	0,7125	0,7125	0,7126	0,0003
354								12,4	-15	11,1	30	3,1	1,34									
356								12,1	-13,8	15	41,4	3,2	0,62									
368	RM^1	13,6	-17	14,7	38,4	3	1,18	12,8	-14,3	14,4	39	3,2	18,13	0,8	2,7	-10,3	-1,6	0,7109	0,711	0,7107	0,7109	0,0003
403	LM_1 /Crown							11,7	-12,1	11,4	31,1	3,2	29,45			-13,5	-1,9	-	-	-	-	-
406								12,4	-15,5	12,9	34,8	3,1	30,33									
407	LM^1	12	-10,2	17,5	52,4	3,5	2,24	13,7	-14,9	10,3	27,9	3,2	27,09			-1,2	-2	0,713	0,7131	0,7128	0,7129	0,0003
411								13,7	-15,2	12,1	34,6	3,3	5,22									
416	LM^1	14	-14,8	15,1	42,4	3,3	5,63	12,4	-15,6	11,7	33,6	3,3	0,5	1,6	0,8	-8,2	-1,3	0,7113	0,7113	0,7113	0,7113	0
442	RM^2	13,3	-17,1	14,5	40,2	3,2	3,76	13,2	-16,6	16,6	30,8	3,1	1,14	0,1	0,5	-1,4	-3,2	0,7129	0,7127	0,7128	0,7128	0,0002
444								12,4	-13,9	14	38,5	3,2	0,64									
452	LM_2	13,4	-12,4	12,6	34	3,2	1,69	13,5	-14,4	12,2	32,2	3,2	4,86	0,1	2	-8,6	-2,1	0,7139	0,7139	0,7143	0,714	0,0004
454	LC^1/Root LI^2	13,1	-12,6	15	41,4	3,2	7,62	13,5	-15	11,9	31,9	3,1	6,07	0,4	2,4	-	-	0,7118	0,7111	0,7116	0,7115	0,0007
564	LI_2	11,9	-7,7	13,9	37,4	3,1	2,2	11,8	-13,1	13,8	37,1	3,1	1,26	0,1	5,4	-1,4	-1,5	0,7178	0,7168	0,7174	0,7173	0,001

573	LI_2	13,6	-15,3	12,5	34,2	3,2	5,61	12,7	-15,6	12	32,3	3,1	0,47	0,9	0,3	-6,4	-2,1	0,7124	0,7123	0,7123	0,7123	0,0001
574	RM_1	11,1	-17,4	9,7	26,8	3,2	5,38	10,7	-16,2	10,7	29,9	3,3	33,04	0,4	1,2	-9,1	-1,4	0,7164	0,7159	0,7162	0,7162	0,0005
604								12,7	-16	13,2	36	3,2	48,13									
611a	RI_1	14,7	-14,4	14,8	41,2	3,2	1,86	12,9	-15,2	12,7	34,3	3,1	3,94	1,8	0,8	-9,4	-1,7	-	-	-	-	-
611b	RI^1	15,4	-12,4	10,8	29,9	3,2	5,66									-5,8	-2	0,7098	0,7097	0,7097	0,7097	0,0001
623								11,5	-13,8	12,4	33,7	3,2	2,33									
625	LM_1	13,2	-12,8	14,4	39,7	3,2	4,79	13,4	-14,7	12	32	3,1	4,25	0,2	1,9	-1,2	-3	0,7141	0,7141	0,7142	0,7142	0,0001
626								12,5	-15,6	11,7	31,5	3,1	7,31									
627								13,3	-16,3	12,2	32,7	3,1	2,15									
628	$L1_2$	12,5	-16,5	13,5	37	3,2	0,85	12,2	-16,5	12,4	33	3,1	3,59	0,3	0	-9,2	-2,6	0,7127	0,7127	0,7123	0,7126	0,0004
629	LI^1	12,8	-15,6	15,6	43,4	3,2	3,88	12,3	-16,4	11	29,7	3,1	5,63	0,5	0,8	-8,1	-2,3	0,7136	0,7133	0,7131	0,7133	0,0005
634								10,6	-15,5	14,8	39,8	3,1	1,44									
636	RI_1	13,4	-14,6	11,8	33,8	3,3	3,32	13,3	-13,9	11,9	32	3,1	5,96	0,1	0,7	-5,8	-1,4	0,7129	0,713	0,7127	0,7129	0,0003
638								10,3	-16,5	13	36,2	3,2	0,61									
641	RM_1	13,2	-17,8	13,8	38,5	3,3	9,43	12,5	-17,1	11,6	31,2	3,1	3,73	0,7	0,7	-12,1	-5,3	0,7107	0,7108	0,7106	0,7107	0,0002
671	RM^1	15,2	-15,9	12,1	32,7	3,2	0,61	14,3	-16,8	11,6	31,5	3,2	3,89	0,9	0,9	-10,8	-2,6	0,7124	0,7126	0,7127	0,7126	0,0003
672	RI_1	12,2	-14,6	15,5	43	3,2	5,56	9,7	-16,8	11,6	31,9	3,2	5,23	2,5	2,2	-4,6	-2,7	0,713	0,7127	0,7127	0,7128	0,0003

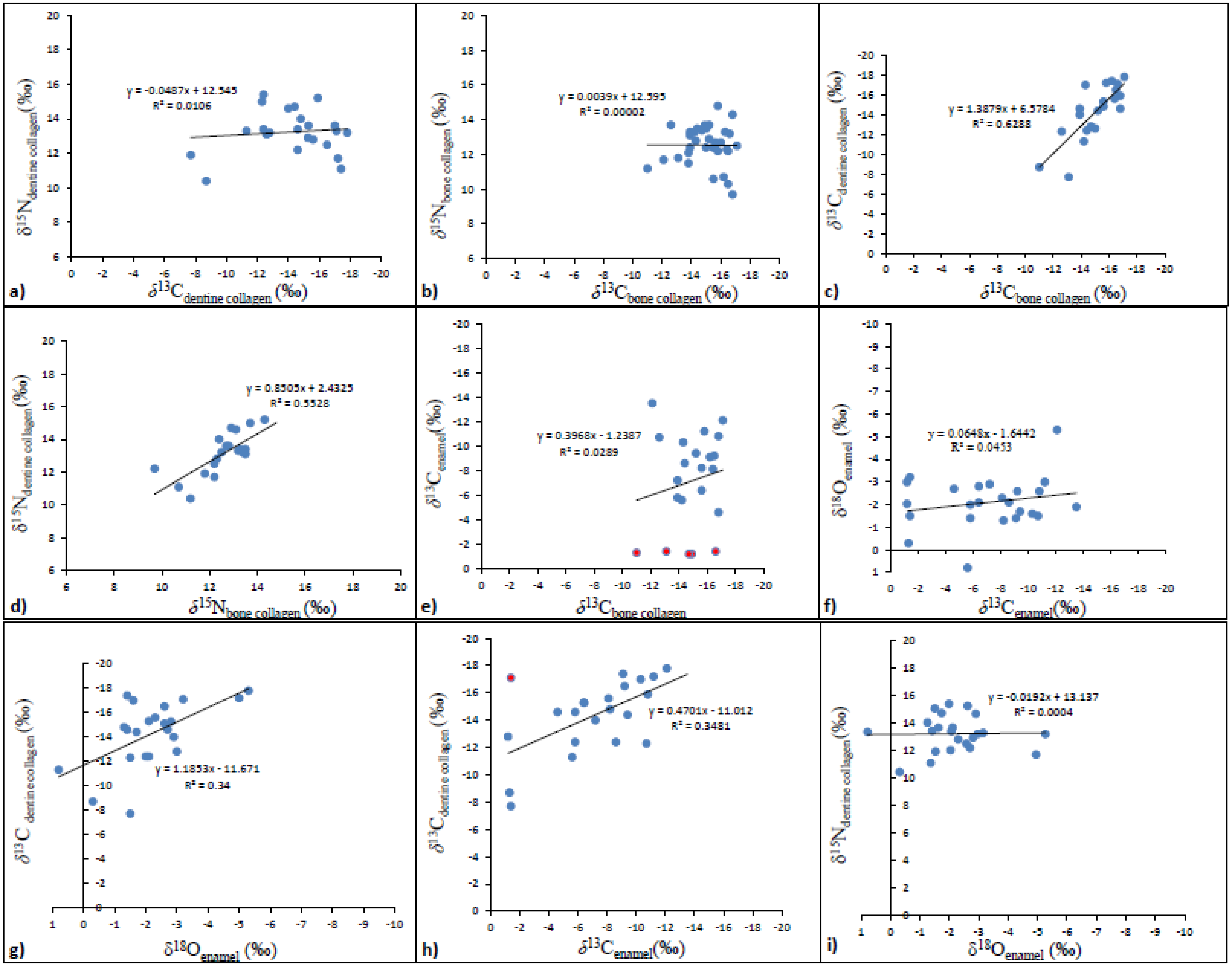


Figure 9: Scatter plots of the various isotopic variables plotted against each other, with linear regression equations and correlation coefficients.

4.2. $\delta^{15}N_{\text{dentine collagen}}$ vs. $\delta^{13}C_{\text{dentine collagen}}$

Figure 9 (a) above shows the scatter plot of $\delta^{15}N_{\text{dentine collagen}}$ against $\delta^{13}C_{\text{dentine collagen}}$ for all individuals in this study with teeth available for analysis. $\delta^{15}N_{\text{dentine collagen}}$ values vary from 10.4 to 15.4 ‰, with a range of 5 ‰ (n=23). The mean is 13.2 ±1.2‰ and the median is 13.3‰. $\delta^{13}C_{\text{dentine collagen}}$ values vary from -17.8 to -7.7‰ which spans a range of 10.1‰ (n=23); the mean value is -14.2 ±2.7‰ and the median is -14.6‰. Two points (at -7.7 and -8.7‰) are markedly more positive than the rest. If these two are excluded, the values vary between -17.8 to -11.3‰, i.e., a range of 6.5‰. The individual with the most positive $\delta^{13}C_{\text{dentine collagen}}$ value (UCT 564/24546 at -7.7‰) consumed a C_4-based diet while the individual with the most negative $\delta^{13}C_{\text{dentine collagen}}$ (UCT 641/24396 at -17.8‰) consumed a largely C_3-based diet. Their $\delta^{15}N_{\text{dentine collagen}}$ values are relatively similar, at 11.9‰ and 13.2‰ respectively. The linear regression equation is y= -0.0487x + 12.545, with an R^2 value of 0.0106, showing no relationship between the variables. This implies that the factor/s driving the variation in $\delta^{13}C$ are not driving variation in $\delta^{15}N$.

4.3. $\delta^{15}N_{\text{bone collagen}}$ vs. $\delta^{13}C_{\text{bone collagen}}$

Figure 9 (b) shows a scatter plot of $\delta^{15}N_{\text{bone collagen}}$ against $\delta^{13}C_{\text{bone collagen}}$ for all individuals in this study. $\delta^{15}N_{\text{bone collagen}}$ values vary from 9.7 to 14.8‰ with a range of 5.1 ‰ (n=35). The mean is 12.5 ± 1.1‰ and the median is 12.5‰. $\delta^{13}C_{\text{bone collagen}}$ values vary from -17.1 to -11.0‰ which spans a range of 6.1‰ (n=35); the mean value is -15.0 ± 1.4‰ and the median is -15.2‰. The linear regression equation is y= 0.0039x +12.595 with R^2 value of 0.00002. The individual with the most positive $\delta^{13}C$ value (UCT 136/24510 at -11.0‰) consumed a mixed C_4 and C_3 based diet while the individual with the most negative $\delta^{13}C$ (UCT 641/24527 at -17.1‰) consumed a mostly C_3 based diet. The $\delta^{15}N$ values for these two individuals are 11.2 and 15.3‰ respectively.

4.4. $\delta^{13}C_{\text{dentine collagen}}$ vs. $\delta^{13}C_{\text{bone collagen}}$

Figure 9 (c) is a plot of $\delta^{13}C_{\text{dentine collagen}}$ against $\delta^{13}C_{\text{bone collagen}}$. $\delta^{13}C_{\text{dentine collagen}}$ values vary from -17.8 to -7.7‰ with a range of 10.1‰ (n=23). The mean is -14.2 ±2.7‰ and the median is -14.6‰. $\delta^{13}C_{\text{bone collagen}}$ values vary from -17.1 to -11.0‰ which spans a range of 6.1‰ (n=35); the mean value is -15.0 ±1.4‰ and the median is -15.2‰. The linear regression equation is y= 1.3879x +

6.5784, with R^2 value of 0.6288. This shows a relatively strong relationship between $\delta^{13}C_{dentine\ collagen}$ and $\delta^{13}C_{bone\ collagen}$, as might be expected for samples from the same individual.

4.5. $\delta^{15}N_{dentine\ collagen}$ vs. $\delta^{15}N_{bone\ collagen}$

Figure 9 (d) shows the scatter plot of $\delta^{15}N_{dentine\ collagen}$ against $\delta^{15}N_{bone\ collagen.}$ $\delta^{15}N_{dentine\ collagen}$ values vary from 10.4‰ to 15.4‰ with a range of 5‰ (n=23). The mean value is 13.2 ±1.3‰ and the median is 13.3‰. $\delta^{15}N_{bone\ collagen}$ values vary from 9.7‰ to 14.8‰ which spans a range of 5.1‰ (n=35); the mean value is 12.5 ±1.1‰ and the median is 12.5‰. The linear regression equation is y= 0.0.8505x + 2.4325, with an R^2 value of 0.5528. $\delta^{15}N$ values appear to be relatively consistent over the lifetimes of individuals in this study, at least when compared with $\delta^{13}C$ values.

4.6. $\delta^{13}C_{enamel}$ vs. $\delta^{13}C_{bone\ collagen}$

Figure 9 (e) shows the scatter plot of $\delta^{13}C_{enamel}$ against $\delta^{13}C_{bone\ collagen}$. $\delta^{13}C_{enamel}$ values range from -13.5 to -1.2‰ with a range of 12.3‰ (n=24). The mean is –7.1 ±3.7‰ and the median is -7.26‰. $\delta^{13}C_{bone\ collagen}$ values vary from -17.1 to -11.0‰ with a difference of 6.1‰ (n=35); the mean value is -15.0 ±1.4‰ and the median is -15.2‰. The linear regression equation is y = 0.3960x -1.2387 with $R^2 = 0.0289$. If the five points (red) with very positive $\delta^{13}C_{enamel}$ values are removed the linear regression equation is y=0.237x -12.469 with R^2=0.0212. As reflected in the R^2 value, the data points are very scattered, indicating a weak relationship. This will be discussed further in the next chapter.

4.7. $\delta^{18}O_{enamel}$ vs. $\delta^{13}C_{enamel}$

Figure 9 (f) is a scatter plot of $\delta^{18}O_{enamel}$ against $\delta^{13}C_{enamel.}$ $\delta^{18}O_{enamel}$ values vary from -5.3 to 0.8‰ with a range of 6.1‰ (n=24). The mean is -2.1 ±1.1‰ and the median is -2.1‰. $\delta^{13}C_{enamel}$ values vary from -13.5 to -1.2‰ which spans a range of 12.3‰ (n=24); the mean value is -7.1 ±3.7‰ and the median is -7.6‰. The linear regression equation is y= 0.0648x – 1.6442, with $R^2 = 0.0453$. This indicates that there is negligible correlation between the two variables.

4.8. $\delta^{13}C_{enamel}$ vs. $\delta^{13}C_{dentine\ collagen}$

Figure 9 (g) shows the scatter plot of $\delta^{13}C_{dentine\ collagen}$ against $\delta^{13}C_{enamel.}$ $\delta^{13}C_{dentine\ collagen}$ values vary from -17.8 to -7.7‰ which spans a range of 10.1‰ (n=23); the mean value is -14.2 ±2.7‰ and the median is -14.6‰. $\delta^{13}C_{enamel}$ values range from -13.5 to -1.2‰ with a range of 12.3‰ (n=24); the mean value is -7.1 ±3.7‰ and the median is -7.6‰. The linear regression equation is y= 0.4701x + 11.012, with R^{2} = 0.3481; if one outlying point, UCT 442 ($\delta^{13}C_{dentine\ collagen}$ = -17.1‰, $\delta^{13}C_{enamel}$ = -1.4‰) is removed, the R^2 value increases to 0.5567 with the regression equation of y= 0.623x - 9.697. This correlation is much better than that seen in the previous plot. The points lying far from the regression line may indicate isotopic differences between carbon going into the dentine collagen and the enamel, probably because of dietary routing. This topic will be discussed further in the next chapter.

4.9. $\delta^{13}C_{dentine\ collagen}$ vs. $\delta^{18}O_{enamel}$

Figure 9 (h) shows a scatter plot of $\delta^{13}C_{dentine\ collagen}$ against $\delta^{18}O_{enamel}$. $\delta^{13}C_{dentine\ collagen}$ values vary from -17.8 to -7.7‰ which spans a range of 10.1‰ (n=23); the mean value is -14.2 ±2.6‰ and the median is -14.6‰. $\delta^{18}O_{enamel}$ values vary from -5.3 to 0.8‰ with a range of 5‰ (n=24). The mean is -2.2 ±1.2‰ and the median is -2.1‰. The linear regression equation is y= 1.1853x – 11.671, with R^{2} = 0.34, indicating a moderate relationship.

4.10. $\delta^{15}N_{dentine\ collagen}$ vs. $\delta^{18}O_{enamel}$

Figure 9 (i) shows a scatter plot of $\delta^{15}N_{dentine\ collagen}$ against $\delta^{18}O_{enamel}$. $\delta^{15}N_{dentine\ collagen}$ values vary from 10.4‰ to 15.4‰ with a range of 5‰ (n=24). The mean value is 13.2 ±1.3‰ and the median is 13.2‰. $\delta^{18}O_{enamel}$ values vary from -5.3 to 0.8‰ with a range of 5‰ (n=24). The mean is -2.2 ±1.2‰ and the median is -2.0‰. The linear regression equation is y=0.0192x + 13.137, with R^2 = 0.0004, indicating no relationship.

4.11. Strontium

$^{87}Sr/^{86}Sr$ was measured in 22 teeth, where possible early forming teeth but due to limitations in availability, the teeth analyzed include first incisors (n=8), second incisors (n=3), canines (n=2) and first (n=7) and second (n=2) molars. Comparison of analysis lines approximately parallel to the occlusal surface, in the middle of the tooth and near the dentine collagen-enamel junction shows possible variation during the growth of the tooth. Mean $^{87}Sr/^{86}Sr$ per tooth ranged from 0.7097 to 0.7188 (Table 4). The intra-tooth ranges varied from 0 for UCT 416/24385 to 0.0010 for UCT 564/24546. The analytical uncertainty associated with these measurements is 0.0003. Only two teeth showed variation greater than 0.0006 (Fig. 10). These $^{87}Sr/^{86}Sr$ ratios will be further discussed in the following chapter in conjunction with the $\delta^{13}C$ and $\delta^{18}O$ values for enamel and the results for bone and dentine collagen.

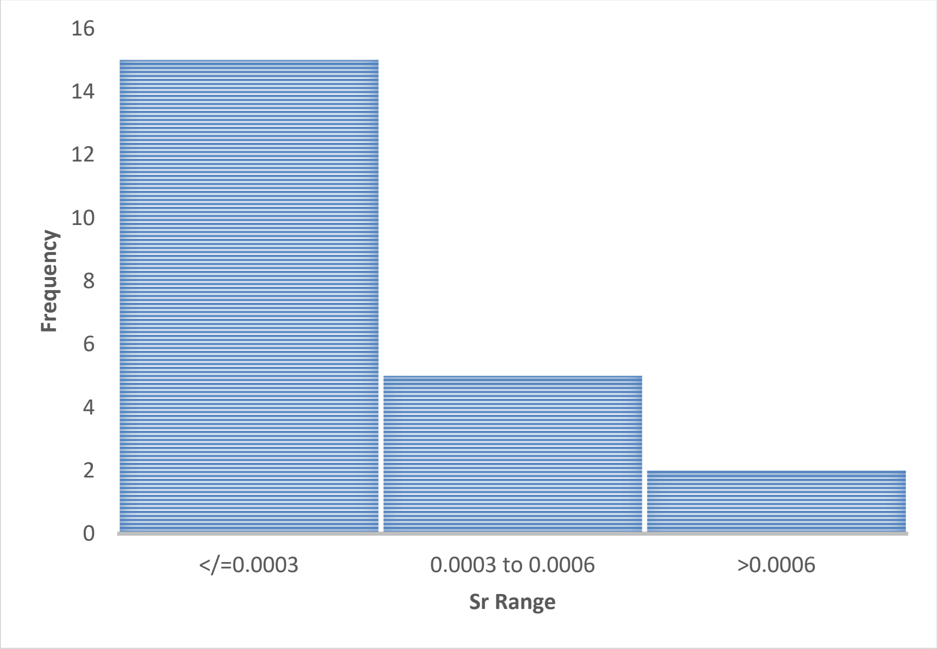


Figure 10: Histogram showing the distribution of within-tooth $^{87}Sr/^{86}Sr$ ranges.

CHAPTER FIVE: DISCUSSION AND CONCLUSIONS

5.1. Introduction

The goal of this study was to characterize the range of isotopic variation in forensic cold cases in the University of Cape Town Human Skeletal Repository, and where possible to determine the dietary patterns of individuals through isotopic analysis of the human skeletal remains. This chapter will interpret the results presented in Chapter 4 and compare them with similar studies conducted elsewhere in the world. The chapter will also pay attention to intra-individual isotopic variation, to assess dietary consistency or change. Substantial dietary shifts during life may indicate mobility of the individuals.

As reported in Chapter 4, the range of values for $\delta^{13}C_{\text{dentine collagen}}$ is 10.1‰ (-17.8 to -7.7‰), compared with only 6.1‰ for $\delta^{13}C_{\text{bone collagen}}$ (-17.1 to -11.0‰). The larger range in $\delta^{13}C_{\text{dentine collagen}}$ indicates more diversity in diets in early life compared with later life, although the difference lies largely in the two individuals with the most positive $\delta^{13}C_{\text{dentine collagen}}$ values of -7.7 and -8.7‰ respectively. These individuals might have originated from rural areas, where they consumed mainly C_4-based diets.

Of the 22 individuals with both rib and teeth samples, fourteen have $\delta^{13}C_{\text{dentine collagen}}$ more positive than $\delta^{13}C_{\text{bone collagen}}$, while seven have $\delta^{13}C_{\text{dentine collagen}}$ more negative than $\delta^{13}C_{\text{bone collagen}}$. One individual (UCT 628) has $\delta^{13}C_{\text{dentine collagen}}$ and $\delta^{13}C_{\text{bone collagen}}$ values that are constant at -16.5‰.

Figure 11(a) shows the distribution of $\delta^{13}C_{\text{dentine collagen}}$ - $\delta^{13}C_{\text{bone collagen}}$ for the same individual. UCT 628/24393 (LI_2) shows no difference ($\delta^{13}C_{\text{dentine collagen}}$ = -16.5‰, $\delta^{13}C_{\text{bone collagen}}$ = -16.5‰) and UCT 352/24399 (RC^1) a negligible difference of 0.1‰ ($\delta^{13}C_{\text{dentine collagen}}$ = -14.0‰, $\delta^{13}C_{\text{bone collagen}}$ = -13.9‰), showing that the individuals consumed an isotopically consistent diet across the period of formation of these tissues. UCT 564/24546 (LI_2) shows the largest difference, with $\delta^{13}C_{\text{dentine collagen}}$ -7.7‰ and $\delta^{13}C_{\text{bone collagen}}$ -13.1‰, i.e., a difference of -5.4‰. These values show a shift from a very strong C_4 to a more mixed C_4- and C_3-based diet as the individual grew older. In southern Africa, the C_4-based foods are likely to be maize meal and meat from animals that consumed C_4 grasses. Subsistence farmers in rural areas tend to consume strongly C_4-based diets, and to rely heavily on maize meal. Urban populations such as those residing in the Greater Cape Town area tend to consume a greater variety of foods, including (in addition to maize meal), wheat, potatoes, rice, chicken, beef, and fish, leading to δ^{13}C values intermediate between the C_4 and C_3 endpoints. The very marked dietary shift seen in UCT 564/24546, who consumed more C_4 foods in early life and transitioned to a mix of C_4 and C_3 foods later, would be consistent with a move from a rural area to Cape Town.

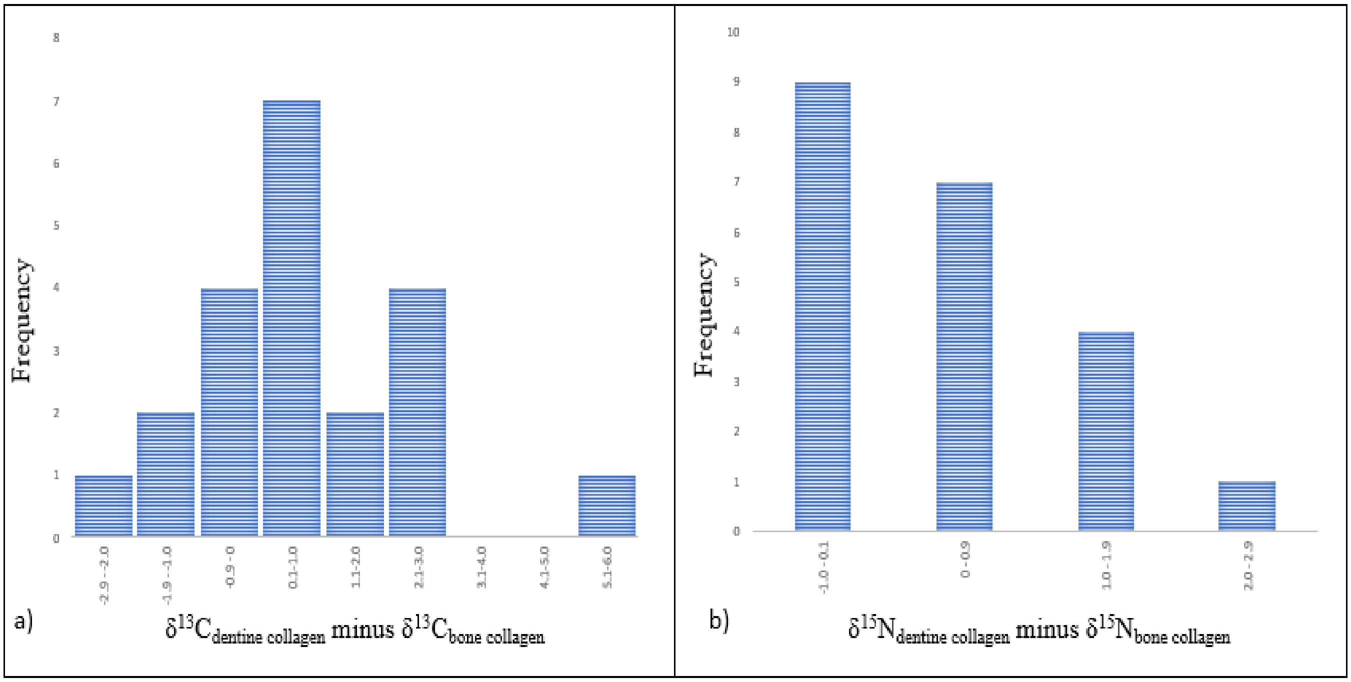


Figure 11: Histograms showing the distributions of (a) $\delta^{13}C_{\text{dentine collagen}}$ - $\delta^{13}C_{\text{bone collagen}}$; (b) $\delta^{15}N_{\text{dentine collagen}}$ - $\delta^{15}N_{\text{bone collagen}}$.

The range of values for $\delta^{15}N_{\text{dentine collagen}}$ is 5‰ (10.4 – 15.4‰), compared with 5.1‰ (9.7 – 14.8‰) for $\delta^{15}N_{\text{bone collagen}}$, a difference of only 0.1‰. The dietary shifts mentioned above, such as expanding from mostly maize meal to include wheat, potatoes and/or rice, appear not to have significantly impacted consumer δ^{15}N values, probably because these are not protein-rich foods. Figure 11 (b) shows a histogram of the distribution of differences between $\delta^{15}N_{\text{dentine collagen}}$ and $\delta^{15}N_{\text{bone collagen}}$ for the same individual.

Fifteen of the 22 individuals with both rib and dentine samples have $\delta^{15}\text{N}_{\text{dentine collagen}}$ more positive than the $\delta^{15}\text{N}_{\text{bone collagen}}$, while 6 have $\delta^{15}\text{N}_{\text{dentine collagen}}$ more negative than $\delta^{15}\text{N}_{\text{bone collagen}}$. The individual UCT233/24383 exhibits consistent $\delta^{15}\text{N}_{\text{dentine collagen}}$ and $\delta^{15}\text{N}_{\text{bone collagen}}$ values, both measuring 13.3‰. This suggests a stable dietary pattern for this individual. Only one individual (UCT 672) shows a difference greater than 2‰ between dentine and bone collagen, with $\delta^{15}\text{N}_{\text{bone collagen}}$ value of 9.7‰ and $\delta^{15}\text{N}_{\text{dentine collagen}}$ of 12.2‰, which is an offset of 2.5‰. The remaining individuals have offsets that are less than 2‰ and therefore cannot be interpreted as evidence of dietary change (See Table 5).

In this thesis, I limit the discussion of intra-skeletal differences to individuals that show a variation of 2‰ or more, following DeNiro & Schoeninger, (1983), Cox *et al.,* (2001), and Plomp *et al.,* (2020). Intra-skeletal differences ≥ 2‰ are likely to indicate that there was a change in diet and/or place of origin. Differences of less than 2‰ may reflect these types of change but may also result from metabolic variation. Table 5 shows the seven individuals with intra-skeletal differences in $\delta^{13}\text{C}_{\text{collagen}} \geq 2‰$, one (UCT 672) also showing a difference of ≥ 2‰ in $\delta^{15}\text{N}$. In all but one, $\delta^{13}\text{C}_{\text{dentine collagen}}$ values are more positive than the $\delta^{13}\text{C}_{\text{bone collagen}}$. The exception is UCT 368 with $\delta^{13}\text{C}_{\text{bone collagen}}$ 2.7‰ more positive than $\delta^{13}\text{C}_{\text{dentine collagen}}$.

Table 5: Individuals with intra-skeletal $\delta^{13}\text{C}_{\text{collagen}}$ and $\delta^{15}\text{N}_{\text{collagen}}$ differences ≥ 2‰.

UCT Accession $\text{N}^{\text{O.}}$	**$\delta^{13}\text{C}_{\text{dentine collagen}}$ (‰)**	**$\delta^{13}\text{C}_{\text{bone collagen}}$ (‰)**	**Difference (‰)**	**$\delta^{15}\text{N}_{\text{dentine collagen}}$ (‰)**	**$\delta^{15}\text{N}_{\text{bone collagen}}$ (‰)**	**Difference (‰)**	**Tooth Sampled**	**Age of dentine formation (years)**
136	-8.7	-11.0	2.3	10.4	11.2	0.8	LI$_1$	9
233	-11.3	-14.2	2.9	13.3	13.3	0	RI$_1$	9
368	-17.0	-14.3	-2.7	13.6	12.8	0.8	RM^1	9-10
452	-12.4	-14.4	2.0	13.4	13.5	-0.1	LM$_2$	14-15
454	-12.6	-15.0	2.4	13.1	13.5	-0.4	Root LI^2	11
564	-7.7	-13.1	5.4	11.9	11.8	0.1	LI$_2$	10
672	-14.6	-16.8	2.2	12.2	9.7	2.5	RI$_1$	9

It should be noted that δ^{13}C and δ^{15}N values for bone and dentine collagen are directly comparable with one another, i.e. there is no systematic offset between the two resulting from metabolic factors (France & Owsley 2015; Salazar-Garcia et al., 2014). Comparison of $\delta^{13}C_{\text{bone collagen}}$ with $\delta^{13}C_{\text{dentine collagen}}$ of the same individuals in this study is therefore equivalent to the comparison of $\delta^{13}C_{\text{bone collagen}}$ for multiple skeletal elements as carried out by Berg et al., (2022), bearing in mind that bone and dentine collagen formed at different times of life. Some of the differences reported here between $\delta^{13}C_{\text{bone collagen}}$ and $\delta^{13}C_{\text{dentine collagen}}$ are large. Berg *et al.,* (2022) (incorporating earlier work by Olsen *et al.,* (2014), Clark *et al.,* (2017), and Fahy *et al.,* (2017)) proposed that when two skeletal elements have $\delta^{13}C_{\text{collagen}}$ values that differ by more than 0.95‰ (or $\delta^{15}N_{\text{collagen}}$ that differ by more than 1.35‰), they can confidently be considered to derive from different individuals. Results from this study show that $\delta^{13}C_{\text{collagen}}$ within a single individual can vary by up to 5.4‰, and $\delta^{15}N_{\text{collagen}}$ by up to 2.5‰. Moreover, one-third (7/21) of individuals for whom both dentine and rib collagen were analyzed showed differences in $\delta^{13}C \geq 2$‰. This is probably largely due to the nature of South African diets, with maize a particularly important food for rural communities, but less so amongst urban dwellers. The same may be true in other parts of the world.

Cox & Sealy (1997) reported a difference of 7.5‰ in $\delta^{13}C_{\text{collagen}}$ of bone from different parts of the cranium (the frontal region compared with sinus bone) of an individual enslaved and transported from East Africa to the Cape. That study did not report collagen quality indicators, as is the norm today. Nevertheless, thresholds for assessing how many individuals are represented in mixed, commingled assemblages clearly need to be sensitive to context. The results reported here indicate the need to assess intra-skeletal differences in stable isotope ratios in the context of the communities from which they originated.

As reported in Chapter 4, the $\delta^{13}C_{\text{enamel}}$ values in this study show a range of 12.3‰ (-13.5 to -1.2‰). This is more than twice as large as the range for $\delta^{13}C_{\text{bone-collagen}}$ at 6.1‰ (-17.1 to -11.0‰). The scatter plot for $\delta^{13}C_{\text{enamel}}$ versus $\delta^{13}C_{\text{bone-collagen}}$ shows that there is no correlation between the two, with R^2 value of 0.0331 (Fig. 9e). This R^2 increases to 0.424, if three outliers are excluded (UCT105, UCT403, and UCT442). However, even in that case, the correlation between the two remains unexpectedly weak: Loftus & Sealy (2012) reported an R^2 value of 0.71 (after removing two outliers) for a similar plot of $\delta^{13}C_{\text{enamel}}$ versus $\delta^{13}C_{\text{bone-collagen}}$ for Cape Coastal hunters and herders. France & Owsley (2013) reported R^2 of 0.87 for $\delta^{13}C_{\text{bone bioapatite}}$ versus $\delta^{13}C_{\text{bone-collagen}}$ in a historical sample from the United States.

There are two possible reasons for a poor correlation between $\delta^{13}C_{\text{enamel}}$ and $\delta^{13}C_{\text{bone-collagen.}}$ One is that different foods may contribute differentially to the formation of enamel vs. collagen. Protein foods are preferentially routed to protein tissues such as collagen, while carbohydrate foods contribute more to tooth enamel and bone carbonates (Lee-Thorp et al., 1989; Ambrose and Norr, 1993; Howland et al., 2003). Carbohydrate-rich foods such as maize may therefore show more strongly in $\delta^{13}C_{\text{enamel}}$ than $\delta^{13}C_{\text{collagen}}$. The other is the fact that enamel forms in childhood, whereas bone remodels throughout life, thus averaging diet consumed over a longer period.

The correlation between $\delta^{13}C_{\text{enamel}}$ and $\delta^{13}C_{\text{dentine-collagen}}$ is stronger than between $\delta^{13}C_{\text{enamel}}$ and $\delta^{13}C_{\text{bone-collagen}}$. $R^{2\,=}$ 0.3545, and increases to 0.5722 if one outlying point (UCT 442 with $\delta^{13}C_{\text{dentine collagen}}$ = -17.1‰, $\delta^{13}C_{\text{enamel}}$ = -1.4‰) is removed. Enamel and dentine form at approximately the same time, so the closer correlation between these two (compared with $\delta^{13}C_{\text{enamel}}$ and $\delta^{13}C_{\text{bone-collagen}}$) shows that at least some of the variability in the enamel-bone collagen plot is likely to be due to changes in peoples' diets over their lifetimes.

Even so, the plot of $\delta^{13}C_{\text{enamel}}$ against $\delta^{13}C_{\text{dentine-collagen}}$ has a much lower R^2 value than those reported by Loftus & Sealy (2012) and France & Owsley (2013). This weak correlation is probably the result of ^{13}C enriched carbohydrate foods showing up more strongly in $\delta^{13}C_{\text{enamel}}$ compared with $\delta^{13}C_{\text{bone-collagen}}$ (*cf.* Ambrose *et al*., 1997).

Mean $\delta^{18}O_{\text{enamel}}$ is -2.2±1.2‰, with a range of 6.1‰ (-5.3 to 0.8‰) (n=22). These values tend to be slightly more negative than those reported for contemporary wild fauna from the same region, for which mean $\delta^{18}O_{\text{enamel}}$ was 0.0 ± 2.4‰ with a range of 9.0‰ (-4.3 to 4.7) (n=23) (Luyt 2017). This slight bias may reflect differential reliance on small bodies of water susceptible to evaporative enrichment of ^{18}O, and/or the contribution of oxygen from food.

Mean $^{87}Sr/^{86}Sr$ for 20 teeth ranged from 0.7097 to 0.7188. Within-tooth variation ranged from zero (UCT 416/24385) to 0.0010 (UCT 564/24546). There are two individuals, UCT 611b/24391 (mean $^{87}Sr/^{86}Sr$ of 0.7097) and UCT 105/24381 (mean of 0.7099), whose results are relatively near to the value of contemporary seawater and/or recent marine sands, at 0.709175 (McArthur *et al.,* 2001; Müller & Anczkiewicz, 2016). These two individuals may have been part of fishing families that consumed large amounts of seafood. Diets heavily reliant on small fish including bones (such as pilchards) would likely lead to values such as these. The ^{87}Sr/^{86}Sr values in the remaining eighteen samples fall within the range of values documented for the geologies found in Greater Cape Town (summarized in Scott *et al.,* 2020). Low-lying areas covered with geologically recent marine sands have values close to 0.709, while the geologically ancient rocks of the Cape Fold Belt have bioavailable ^{87}Sr/^{86}Sr up to 0.7237. All the values reported in this thesis fall within this range. This is a very wide range, and many other parts of South Africa have similar values. In this study, ^{87}Sr/^{86}Sr is therefore not highly diagnostic of an individual's origins.

Similar values are found in other regions, so ^{87}Sr/^{86}Sr analyses cannot be definitive markers of origin. They would, however, be useful in distinguishing immigrants to Cape Town from areas with highly radiogenic values such as ancient geologies in other parts of South Africa (Copeland *et al.,* 2011, Fowler *et al.,* 2020) or elsewhere in south/central Africa (House *et al.,* 2021, Wang *et al.,* 2023). Volcanic regions such as those in East Africa have low ^{87}Sr/^{86}Sr (Janzen *et al.,* 2020; Tucker *et al.,* 2020) that would also be clearly distinguishable from the values measured in this study.

5.2. Limitations

This study of forensic cold cases has some limitations.

The relatively small sample size (n=37, though not all tissues were available for all individuals) means that the study may not have captured the full range of variation amongst the population of Cape Town. There were limitations on what was available to sample, for instance the first molars or incisors were not preserved for some individuals, so other teeth (e.g. second molars) had to be sampled instead. For some individuals, no teeth were available.

The main goal of this project was to characterize the ranges of isotopic variation for forensic cases from the Greater Cape Town area; however, some individuals may have come to the city from further afield, complicating the attempt to try to characterize the ranges for local residents.

Lastly, due to the sensitivity of these collections, it was not possible to access documentary information collected by the police when these individuals were found, nor research results generated by other researchers e.g. on likely ancestry. This would have been helpful in evaluating especially those individuals with unusual isotopic values and large intra-skeletal differences. Collecting such information was beyond the scope of this project. In combination with these other types of evidence, the isotope results reported in this thesis might be helpful in identifying these unidentified deceased individuals.

5.3. Conclusions

The main conclusions of this thesis are as follows:

- The stable isotope ratios measured in the 37 forensic skeletons analysed for this thesis are highly variable.
- δ^{13}C values, in particular, extend across much of the range previously documented for human populations world-wide, due to consumption of varying mixtures of C_4-based and C_3-based foods.
- δ^{15}N and δ^{18}O values are less variable.
- ^{87}Sr/^{86}Sr values for all individuals in this study fall within the range expected for the Greater Cape Town area.
- This study documents within-individual differences of greater than 2‰ for both $\delta^{13}C_{collagen}$ and $\delta^{15}N_{collagen}$. Thresholds for assessing numbers of individuals in co-mingled skeletal assemblages in forensic or other contexts may need to take account of this.

5.4. Recommendations

Combine isotope analysis with other forensic techniques such as DNA analysis, anthropological data, or chemical profiling. Integration of multiple lines of evidence can strengthen the accuracy and reliability of identification.

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