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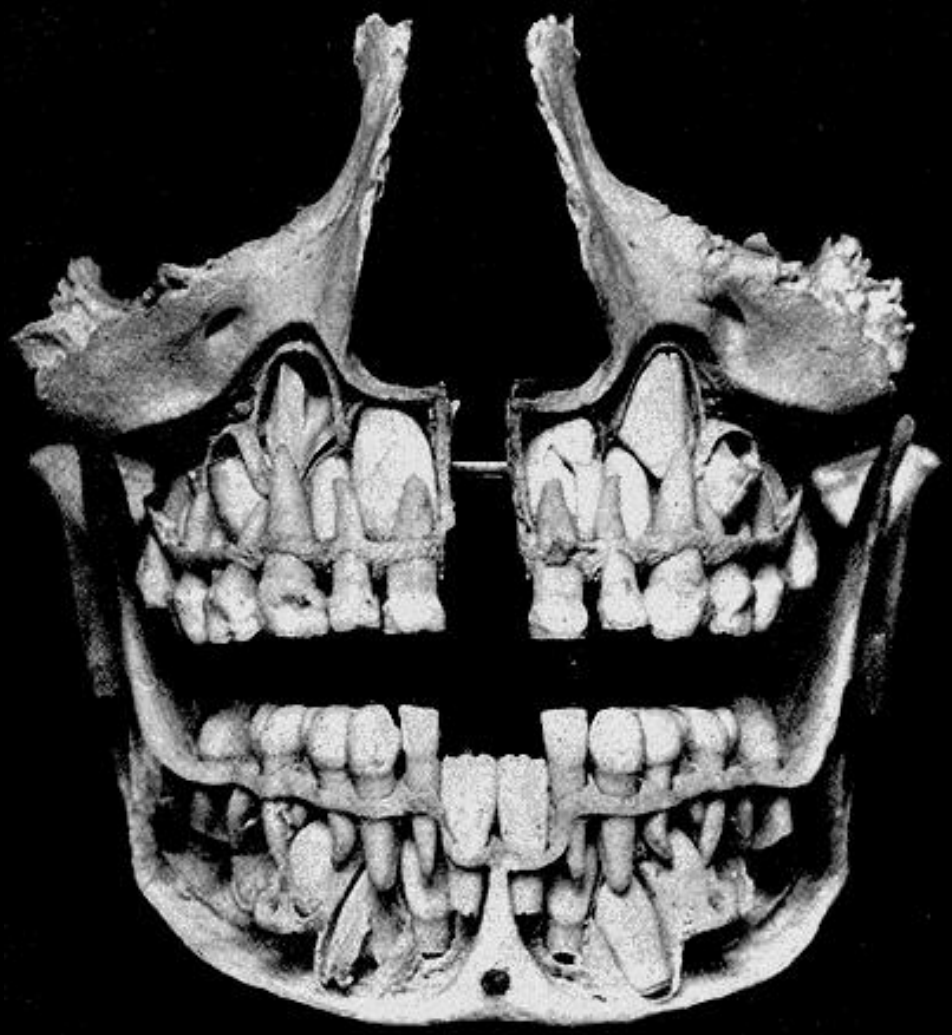

Anna-Maria Mavridou

Cover figure:

Gray, H. and W.H. Lewis, 1918. *Anatomy of the Human Body*. Lea & Febiger, Philadelphia. From: Bartleby. com.

# Tracking breastfeeding and weaning practices in Cuba

Carbon and oxygen isotope analysis in enamel samples from the archaic population of Cueva del Infierno.

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Human Osteoarchaeology

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# 1 INTRODUCTION

Food and water are the essential components of life and growth maintenance. They are considered a major concern in multiple fields of study including archaeology, anthropology and evolutionary biology. Eating behaviors have a central role in most societies, affecting the cultural systems and uncovering social behaviors such as care, healing, beliefs or rituals (Barthes 1979, 22-25; Papathanasiou et al. 2015, 1; Tsutaya and Yoneda 2015, 2). In this regard, ample research has been conducted by osteoarchaeologists in order to bring to light the eating habits of past populations, gaining valuable insights on their ancient lives and cultures.

Food is linked with culture, but it is also critical for a child's survival. In particular, breast milk is responsible for the immune protection of the infant and provides the necessary nutrition for the further growth and development of the child (WHO 1998, 2009). The significance of breastfeeding and weaning practices (BWPs) presents itself in disciplines like epidemiology, gynecology or pediatrics in the contemporary world (WHO 1998, 2009). On the other side of the spectrum, the past can be connected with the present by understanding BWPs in antiquity. breastfeeding and weaning practices can define the general health of past populations, and their study within the fields of archaeology and anthropology sheds light on the degree of influence that infant diets had on the demographics and fertility of these ancient societies (Dettwyler and Fishman 1992; Katzenberg et al. 1996; Tsutaya and Yoneda 2015, 2). A step even further in the past, can indicate a path of evolution of the weaning process itself from hominids to modern human using breastfeeding reconstructions in early human ancestors (Kennedy 2005).

Although of high importance, BWPs vary even in modern times, differentiating due to cultural or economic factors. In most cases, the first nutrition intake of an infant is a mother's breast milk, during breastfeeding. The introduction to liquid or solid food (supplementation) has been suggested to commonly start before six months of age (Izurieta and Larson-Brown 1995; Sellen 2001). This is the first step of the weaning process where nutrients other than breast milk (weaning food) are provided to the child. Weaning completes when the cessation of breastfeeding occurs, mostly around and after the second year of age (Dettwyler

and Fishman 1992; Kennedy 2005; Sellen 2001). Both modern and archaeological studies have shown that the timing and duration of supplementation and weaning differs among populations and cultures (Dettwyler and Fishman 1992; Rivera and Ruel 1997; Wright and Schwarcz 1998). Factors associated with this variation should always be considered when a reconstruction is attempted, as they usually constitute a significantly informative part of the analysis in this kind of study.

## 1.1 Stable isotope analysis in breastfeeding and weaning practices

Stable isotope analysis of skeletal remains offers osteoarchaeologists the unique ability to yield information and reconstruct the diet of an individual, which constitutes not only a means of survival but also cultural and social phenomenon (Papathanasiou et al. 2015, 1). Isotopic signatures have been used since the 1970-1980s to reconstruct breastfeeding and weaning practices using either contemporary or ancient populations (DeNiro and Epstein, 1978,1981; Fildes 1986; Fogel et al. 1989; Roberts et al. 1988). Blood and keratinous tissues are usually used for the study of modern populations, while bone and teeth are used as sample tissues for past populations. The isotopic signatures from skeletal remains of different individuals are used to interpret diets and can then be compared in order to trace trends according to age, culture or status. Different isotopes provide information about different aspects of BWPs. More specifically, carbon indicates can allude to lipid intake coming from breast milk, nitrogen isotopic values provide evidence about breast milk proteins while oxygen ratios evaluate the intake in breast milk water. There are also studies that have used a combination of carbon and sulfur to explore the content of the food used for supplementation (Katzenberg et al. 1996; Tsutaya and Yoneda 2015, 2).

In archaeological studies, the sampling material plays an important role for the accuracy of the research. Samples can be extracted from the organic or the inorganic part of a bone or a tooth. Organic samples are either bone collagen or dentin collagen. Inorganic samples derive from the carbonates of the bone or the inorganic components of the tooth such as dentine carbonate, enamel carbonate or enamel phosphate.

Most of the studies published until present have used bone collagen as a source for isotopic signatures (Bocherens et al. 2007; Burt 2013; Chinique de Armas and Smith 2018; Dupras 2001; Howcroft et al. 2012; Katzenberg et al. 1993; Pearson et al. 2010; Redfern et al. 2012), with carbon and nitrogen values most frequently used (Tsutaya and Yoneda 2015, 4). Teeth on the other hand can be highly informative, especially for BWPs, given the well-studied and established patterns of development and eruption of the deciduous (also called milk or baby teeth) and permanent teeth (AlQahtani et al. 2010; Ubelaker 1989). Differences in diet can be traced during the child's growth, deriving more explicit data about the time period in which the isotopic changes have occurred. The isotopic signatures at the time of formation are retained for every tooth. Moreover, the variation of the isotopic values among samples of different fractions from a tooth can provide more precise chronological comparisons of the isotopic changes. Researchers have used different components of the tooth such as dentin collagen, dentine carbonate, or enamel phosphate in their studies tracing variations (Britton et al. 2005; Buhay et al. 2013; Chinique de Armas et al. 2017; Chinique de Armas and Pestle 2018; White et al. 2004) but overall research is limited to tooth enamel as a source of evidence concerning BWPs (Dupras and Tocheri 2007; Wright and Schwarcz 1998, 1999).

Carbon isotopic signatures provide information about the degree of marine and terrestrial plant consumption by past populations. Within BWPs carbon and nitrogen are most commonly used to track the lipid and protein intake that comes from the breast milk of the mother and examine the content of food used for weaning. On the other hand, oxygen isotopic ratios have been used in archaeological research, mostly focusing on paleoclimate reconstructions (Longinelli 1984) or human mobility (Chenery et al. 2010), rather than dietary studies. The first assumption that oxygen can contribute in tracking breastfeeding and weaning practices was in 1988 (Roberts et al. 1988), with an archaeological application through isotope analysis in 1998 (Wright and Schwarcz 1998). Until present, only a few studies have employed oxygen isotopic signatures to examine BWPs (Britton et al. 2015; White et al. 2004; Williams et al. 2005) and one of the aims of the present study is to contribute to this aspect of oxygen isotope analysis, providing new evidence to the field of study.

## 1.2 Overview of the present study

The main objective of this research is to look for patterns in the isotopic signatures of young children, which could track evidence of changes in their diet during the first years of life. This is achievable by taking multiple samples from the same individual, in different periods of life. This can be accomplished by selecting different teeth that reflect the diet in different periods of life, according to patterns of tooth formation and development (AlQahtani et al. 2010). The periods of infancy selected for study in this research are; *in utero*, the period around birth and the first year of the child's life. The isotopic signatures that correspond to these periods are carried by the deciduous incisors, the deciduous molars and the first permanent molars, respectively. Analyzing these sets of teeth from the same individuals will provide a clearer picture of the mother-infant relationship in terms of the feeding practices followed. The sampling of sequential teeth will lead to time-series of higher resolution, indicating potential trends and exploring inter-individual differences of the isotopic signatures of the archaeological population under study.

For the purposes of this study, the required teeth should be present and preserved in the osteological material available. The population of Cueva Del Infierno (Archaic Cuba) was selected due to the nature of its samples. The majority of the skeletons excavated in this site (83.2%) belonged to children who were under the age of three at the time of death (Ruiz and Beatón 2000, 2-3). The presence of many deciduous teeth and the preservation of mandibular dentition still in occlusion aids the sampling strategy discussed previously. This is a rather rare assemblage available in osteoarchaeology and fits perfectly with the objectives of this research. Furthermore, there are very limited studies in the Caribbean region on the topic of BWPs, and the results of the isotopic values of this population will be novel research.

The approach of this study will focus on dental enamel of subadult individuals. Compared with bone, dental enamel is less vulnerable to degradation and is therefore better preserved (Kohn et al. 1999, 2738). In climates like the tropics of the Caribbean, high temperatures and humidity can lead to earlier degradation of the organic material. Furthermore, even the non-organic component in bone can be prone to alterations under these climate conditions. Conversely, tooth enamel is

generally expected to preserve better, allowing for better preservation of sampleable material (Pestle and Ramos 2018, 274).

The utilization of oxygen isotope analysis in the field of BWPs has shown to be highly informative, and further research can provide insights towards what other questions can be answered. This is especially useful for questions that carbon and nitrogen cannot answer, like the beginning of liquid supplementation in early stages of weaning. There are previous studies in the Caribbean that have utilized stable carbon and nitrogen isotope analysis (Chinique de Armas and Pestle 2018), but there are no published data about oxygen isotopic values in the island of Cuba focusing on childhood dietary reconstructions or BWPs. Furthermore, the carbon isotope analysis will allow comparisons of the subsistence economy of Cueva del Infierno with other Cuban populations. Lastly, carbon and oxygen isotopic values in combination may aid with the understanding of the oxygen isotopic signatures fluctuations during the first years of life, providing different pieces of the same dietary puzzle.

## 1.3 Research questions

The purpose of this research is to explore the application of carbon and oxygen isotopic analysis of human dental enamel to breastfeeding and weaning practices in the archaic Cuban population of Cueva del Infierno. The results of such a study will be of high importance, since the archaeological material available offers a great opportunity for methodological and interpretational improvements. Potential isotopic patterns can be traced that may be characterized by certain breastfeeding practices or possible biases. All of these inquiries will – to a degree - fill gaps of knowledge existing today, providing a framework for fruitful discussion. Moreover, the oxygen isotopic analysis of the multi-sampled individuals will add new, valuable and unique information to previous carbon and nitrogen isotopic studies in other Cuban populations. These new insights will highlight breastfeeding practices, maternal investment and weaning processes adopted from the population under study.

The main research question of this study is whether or not there are specific patterns of isotopic change to be traced in carbon and oxygen signatures of an individual, that follow one's dietary changes from breastfeeding to supplementation, ultimately asking whether different children share the same isotopic trends or not?

Considering the above question, more sub-questions can be formulated in this regard. Is there any kind of correlation between the changes observed in carbon isotopic values with the fluctuations of the oxygen signatures? Is the detection of liquid or solid supplementation possible? Are there any cultural reasons behind the variation? For example, can inferences be made about differences in maternal investment? Or can isotopic variations be linked to osteological differences, like sex or ageatdeath?

Moreover, by examining the carbon isotopic signatures, can we make inferences about the subsistence economy of the people of Cueva del Infierno?

Lastly, by examining water oxygen isotopic values from local drinking water, we can make inferences about their relation to the local water isotopic signatures?

## 1.4 Thesis outline

The present research is organized with the goal of guiding the reader from its questions to the answers through six chapters. The introduction (present chapter) points out the importance and reasoning behind the methods and approach adopted in this study and is followed by the presentation of basic information about stable isotope analysis (Chapter 2). This way, the background theoretical knowledge is provided which will aid in understanding the data later on. Chapter 3 presents all of the necessary information about the materials (the site, the individuals, the samples) and the protocols used in the experiment. The measured isotopic ratios are listed in Chapter 4 and are further discussed in Chapter 5, where the data are interpreted. The final chapter (Chapter 6) provides a brief summary of the research, highlighting all of the significant arguments that led to the answers of the research questions.

# 2 BACKGROUND

Before analyzing the particular case of breastfeeding and weaning practices in ancient Cuba, it is important to consider the basic principles of stable isotope analysis and preceding dietary studies in the Caribbean that have been based on the same methodology. In this section, the theoretical background of stable isotope analysis is explored, together with the main concepts of the sampling methodology. The purpose of the present chapter is to explain the reasons behind the sampling strategy chosen, as well as the general approach to the research problem. This will provide context to the results derived and allow a better understanding of further interpretations.

## 2.1 Stable Isotope Analysis

### 2.1.1 Basic terminology

Chemical elements are atoms which consist of three different kinds of subatomic particles; protons and neutrons in their nuclei and electrons that spin around this core (McNaught and Wilkinson 1997). The term "*isotopes*" describes the different forms of chemical elements that contain a different number of neutrons in their nuclei. Although isotopes have the same number of protons (atomic number, Z) they differ in the number of their neutrons (Faure 1986). The numerical variance of the neutrons results in a relative difference in the atomic masses of the different isotopes, creating "*light*" and "*heavy*" isotopes, without altering the chemical properties of the element to a great degree (Schoeller 1999, 667). The isotopes of an element with an extra neutron (heavy isotope) constitute the rare form of this element, while the light ones are generally more abundant (Schoeller 1999, 667-668). However, the mass variance leads to a differential movement of the element in space (kinetics), an alteration of its reaction rates, or the temperatures in which the element transforms from one state to another (Faure 1986; Meier-Augenstein and Kemp 2012, 5).

There are two categories of isotopes, *stable* and *radioactive*. Isotopes that are characterized as stable are not subjected to decay. This means that the stable isotope ratios are preserved through time, as a result of the stability which is provided by the number of neutrons in their nucleus. Although most isotopes fall in

this category, there are a few that are characterized as unstable due to excess or dearth of neutrons which have the tendency to disintegrate from the nucleus. These elements decay into another element lower in the periodic table and in the process emit radiation; they are called radioactive isotopes. For instance, $^{14}C$ is a radioactive form of carbon, while $^{12}C$ and $^{13}C$ are stable isotopes (Meier-Augenstein and Kemp 2012, 2).

The term *fractionation* refers to the discrimination that can occur during the incorporation of the different isotopes of an element into a living system or an inorganic chemical reaction during biochemical and physical processes in the organism. This means that during these processes there is a preference for which isotope will be used for each biogenic reaction (Schoeller 1999, 668). This can lead to differential isotopic ratios among different tissues or classes of compounds (DeNiro and Epstein 1981, 344; McCutchan et al. 2003).

Lastly, the term isotopic *signature* refers to the consistency of the chemicals ingested from water and food, leaving a chemical signature in the different tissues of the body. Thus, each element under study has its own isotopic fingerprint in different biological material (Wada et al. 1995, 7). Controlled feeding experimental studies have shown that the isotopic signature of different biological tissues reflect the food consumed (DeNiro and Epstein, 1978; Ambrose and Norr 1993; Tieszen and Fegre 1993).

### 2.1.2 The delta (δ) notation

In archaeology, the stable isotopic value of organic and/or inorganic remains is given by a ratio of the relative abundance of the rare (minor abundance, heavier form) to common (major abundance, lighter form) isotope of an element. The isotopic values are calculated with the equation given below (1) (after McKinney et al. 1950, 730), where δ (delta notation) refers to the isotopic ratio which is calculated in parts per mil (‰ or parts per thousand), since the natural abundance differences of the different isotopes are very small. The $R_{sample}$ is the ratio (heavy isotope/light isotope) of the unknown sample and $R_{standard}$ is the ratio (heavy isotope/light isotope) of the reference standard used (McKinney et al. 1950, 730; Fry 2006, 22).

$$\delta\ (‰) = \left(\frac{R(sample)}{R(standard)} - 1\right) \times 1000 \qquad (1)$$

### 2.1.3 Reference standards

There are materials whose isotopic composition has been certified and administered by the International Atomic Energy Agency (IAEA) in Vienna, Austria, or the National Institute of Standards and Technology (NIST), Gaithersburg, USA and they are called reference materials (RMs). These standards are used to verify the accuracy of mass spectroscopy. Because the reference material is not infinite and the high demand for the standard can exhaust the stock (like NBS19) other standards have been calibrated to the same ratios, including one known as VPDB (for Vienna Pee Dee Belemnite or "Vienna PDB") with the reference material IAEA-603, and they have replaced the original standard (certified) (Coplen 1994, 275; Meier-Augenstein and Kemp 2012, 3).

### 2.1.4 Mass spectrometry

The ratio of the rare to common isotopes of an element is estimated by using mass spectrometry, a process where the different isotopes of the element are separated according to their mass-to-charge ratio. More specifically, isotope ratio mass spectrometers (IRMS) use ion sources to charge the atoms and a magnetic field to separate the isotope variants according to their masses (Faure and Mensing 2005, 64; Katzenberg 2008, 420).

An IRMS has four basic components: the inlet system, the ion source, the mass analyzer and a series of ion detectors (Figure 1). Samples are pretreated in the laboratory and then they are introduced in the mass spectrometer as a gas. Combustion of the sample produces water ($H_2O$) and carbon dioxide ($CO_2$); the latter enters the inlet system of the spectrometer. The gas is then led to the ion source. There, some of the gas atoms/molecules are charged (ionized), allowing their control and focus into a beam. Subsequently, this beam is directed to the mass analyzer where it is separated into smaller beams. This separation is due to their different atomic masses. The different isotopes differ in motion under the effect of a magnetic field. This way, the intensity of the respective beams (individual isotope ion beams) can be measured by the ion collectors. The values are reported after measurement of aliquots of both the unknown sample and the standard reference

sample providing a ratio that is calculated by the equation given above (Faure and Mensing 2005, 64-65; Katzenberg 2008, 420-421).

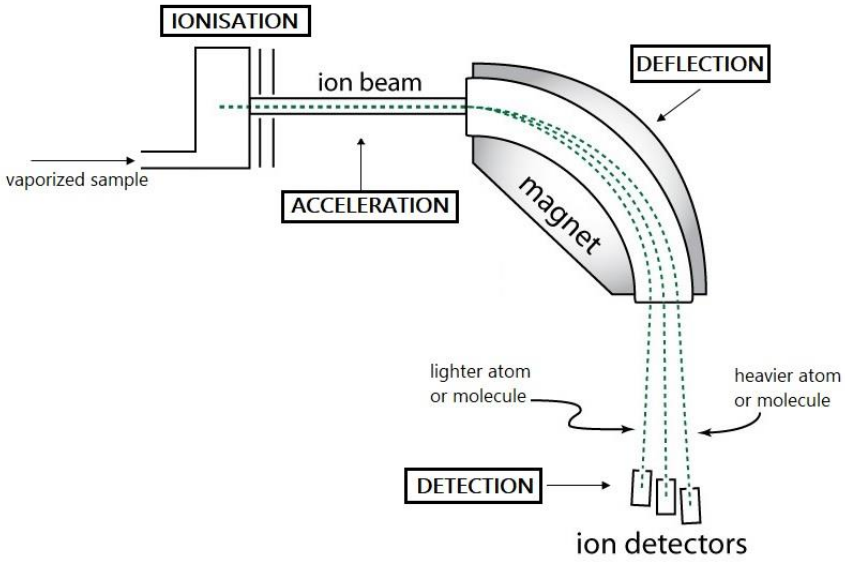



Figure 1 – A Mass Spectrometry Schematic: the arrangement of the basic components of a mass spectrometer. Figure taken from Sercmedia (https://serc.carleton.edu/download/images/9094/massspecschematic.jpg) edited by the author.

### 2.1.5 Isotope Principles

The basis of how isotope analysis works lies on the circulation of the isotopes of elements such as carbon (C), nitrogen (N), oxygen (O) and strontium (Sr) in the biosphere, with fractionation responsible for the variety of their forms and abundance within biological and geochemical systems (Katzenberg 2008, 416; Fry 2006, 41). The chemical transformation of these elements takes place within biogenic systems, creating an isotopic variation according to the different pathways followed. This variation can be traced by archaeologists and biological anthropologists using isotope analysis to gain insights and understand different aspects of past people's lives (Lee-Thorp 2008, 927; Katzenberg 2008, 416). Isotopic circulation and fractionation result in different but characteristic distributions of the isotopic ratios, providing pools with stable ranges for each isotope within the overall reservoir based on space and time (Fry 2006, 41). Furthermore, because the biochemical composition differs among species, but also

among individuals, isotopic ratios vary for different species, individuals or tissues (DeNiro and Epstein 1981, 344; McCutchan et al. 2003)

Different elements can give information for different aspects of past lives. Samples from animals or humans can be used to provide information about the food consumed using stable isotope analysis. For example, carbon and nitrogen isotopes are used widely to reconstruct past diets (paleodiet) (DeNiro and Epstein 1978; DeNiro and Epstein 1981; Ambrose and Norr 1993). Carbon isotopic signatures provide evidence about the proportion of plants consumption in the diet while nitrogen can be used to place the organism under study on the right trophic level within the food pyramid, reflecting the relative origin of the proteins consumed (Wilhelmson 2017, 268). On the other hand, oxygen and strontium are mostly used in mobility studies giving information about the locality of the water or food consumed (Chenery et al. 2010; Eckardt et al. 2009; Laffoon et al. 2013; Sponheimer and Lee-Thorp 2001). Oxygen isotope analysis has been mostly employed in paleoclimatic studies due to the distribution of its isotopes in water (Longinelli 1984). It can also be used in dietary ecology, contributing information on the water found in the human body either consumed as water directly or through food (Kohn 1996; Sponheimer and Lee-Thorp 2001). For the purposes of the present study carbon and oxygen will be further discussed in the following paragraphs.

#### *2.1.5.1 Carbon*

Carbon has two different stable isotopes, $^{13}C$ and $^{12}C$, and one radioactive isotope $^{14}C$, which is widely known for its usage in dating (Walker and Walker 2005, 17-54). As mentioned above, stable isotopes of carbon provide information about the degree of marine and terrestrial plants consumption by past populations (Wilhelmson 2017, 268). The different plants can be categorized in three types, based on the photosynthetic pathway that they use to grow. These are the $C_3$ plants (most fruits, vegetables, nuts or legumes), $C_4$ plants (e.g. sugar, maize or millet) and crassulacean acid metabolism (CAM) plants (such as succulent plants) (Smith and Epstein 1971). Each category has a different abundance of $^{13}C$ and $^{12}C$ providing distinguishable $^{13}C/^{12}C$ isotope ratios. The international standard used to calibrate and calculate $\delta^{13}C$ is the Vienna Pee Dee Belemnite (VPDB) standard (Coplen 1994, 275). While the $\delta^{13}C$ signature of $CO_2$ in the atmosphere lies around

-8‰ (Keeling et al. 2005), $C_3$ plants have $\delta^{13}C$ signatures between -24‰ and -34‰, $C_4$ plants have $\delta^{13}C$ values from -6‰ to -19‰ and CAM plants have $\delta^{13}C$ ratios similar to $C_4$ plants but somewhat overlap both ranges (Boutton 1991, 173-178; Smith and Epstein 1971, 383-384). Information can also be provided by the isotopic values observed between marine and terrestrial plants (Schoeninger and DeNiro 1984), where it has been noticed that marine $\delta^{13}C$ values are less negative by 7-8‰ on average (Boutton 1991, 183; Chisholm et al. 1982, 1131). These variances in ratios among the different categories allow estimations to be made about the proportion of each plant category in past diets.

The composition in carbon isotopes within the biogenic samples reflects the individual's average dietary input, allowing interpretations regarding paleodiet, paleoecology and paleoenvironment of the species under study. Although informative, one should take under consideration the "shift" that is happening between dietary values and ratios in the body due to biochemical processing (and subsequent fractionation) of the food. This "shift" is also greatly dependent on the tissue extracted and analyzed (DeNiro and Epstein 1978, 499-503) and the trophic level of the animal (for example herbivore, carnivore or omnivore) (Krueger and Sullivan 1984, 215-218; Lee-Thorp et al. 1989, 591-595). The possible ranges of the carbon isotopic values in either collagen or apatite of human bone have been synopsized for different possible kinds of diets (Krueger and Sullivan 1984).

#### *2.1.5.2 Oxygen*

There are three stable isotopes of oxygen that can be found in nature: $^{16}O$, $^{17}O$, $^{18}O$. Among them $^{16}O$ is the most abundant (99.762% natural abundance) with $^{18}O$ following (0.2% natural abundance) (Schoeller 1999, 668). As in the case of carbon, the international standard that is used as reference to calibrate and calculate $\delta^{18}O$ values derived from (structural) carbonate is the Vienna Pee Dee Belemnite (V-PDB) (Coplen 1994, 275) using equation (1) given in page 14.

The oxygen isotopic ratios of skeletal tissues correlate with the isotopic values of the water of the area. More specifically, precipitation plays a highly important role for local isotopic signatures. Oxygen is present in different forms and amounts among the components of the earth system (reservoirs). It can be found in the atmosphere either as gas or water vapor, in the oceans as sea water and in ice

sheets as frozen water. Oxygen isotopes transfer through the different reservoirs according to temperature and the difference of their masses. Due to its slightly lighter mass, ($^{16}O$) evaporates more readily from surface water resulting in $^{18}O$ enrichment of sea water. On the other hand, the heavier form ($^{18}O$) precipitates more rapidly out of water vapor. Factors such as temperature, coastal proximity, latitude, altitude and season influence the local oxygen isotopic values by affecting the fractionation of the different isotopes among the earth systems (Dansgaard 1954; Epstein 1956; Gilfillan 1934). For example, clouds are more enriched with $^{16}O$ towards the poles which will fall in the form of snow, while $\delta^{18}O$ changes after temperature fluctuations, in this way providing records of the climate (Dansgaard 1964, 4).

These principles have been used for years by archaeologists to reconstruct past climates and seasonality (Balasse et al. 2003; Bryant et al. 1994). Nevertheless, the phenomenon of fractionation occurs also for oxygen isotopic values within the biogenic system from ingested local water to body water. A slight enrichment of the oxygen isotopic signatures takes place while water is ingested indirectly through food, which does not affect the linear relationship of the two (Bryant and Froelich 1995; Kohn 1996). Furthermore, studies have shown that in cases where $\delta^{18}O$ ratios show spatial variation rather than homogeneity for a region, the values can be used to trace provenance, leading to mobility interpretations (Chenery et al. 2010; Laffoon et al. 2013; Schroeder et al. 2009).

Lastly, and most importantly for the present study, oxygen isotope analysis has also found its usage in paleodiet, where it plays a significant role for tracking breastfeeding and weaning practices in past populations and, more specifically, the water intake from the breast milk (Britton et al. 2015; Tsutaya and Yoneda 2015; Wright and Schwarcz 1998, 1999). Interpretations like this are based on the premise that oxygen isotopic signatures will be enriched in infant tissues during breastfeeding. Specifically, a mother's breast milk will be enriched in the heavier isotope ($^{18}O$) compared to the isotopic composition of the water ingested, due to fractionation. This way, $\delta^{18}O$ ratios of breastfeeding infants show a difference of +2-3‰ during breastfeeding compared to the values of the individual after breastfeeding cessation (complete weaning) (Roberts et al. 1988, 625-627; Wright and Schwarcz 1998).

### 2.1.6 Tissues that can be sampled in stable isotope analysis

Bone and teeth constitute the most commonly sampled tissues in isotope analysis, since calcified tissues are better preserved in the archaeological record. However, there are cases where also keratinous tissues like hair and nails can survive in skeletal remains which can provide valuable isotopic evidence (Lee-Thorp 2008, 926). One should carefully select the right kind of tissue for sampling according to the research questions and availability of sample material. For example, teeth grow during childhood so their values are determined during their formation (Hillson, 2005, 152-153), while bone will reflect the isotopic composition in the years before death (Hedges et al. 2007, 814-815). Furthermore, keratin tissues like hair and nails that grow at faster rates can be used as sequential samples to examine differences within shorter periods, such as a few months before death (Millard 2000; Fogel et al. 1989; Fuller et al. 2006).

#### *2.1.6.1 Bone*

Dry bone is one of the main sample tissues in archaeological isotope analysis. Bone is composed of approximately 30% organic (most of it constitutes of collagen) and 70% inorganic material (mainly hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$)) (Burton 2008, 443-444; Katzenberg 2008, 416; Triffit 1980, 45-82). Bone cells are called osteons and every second 2-4% of them are being replaced in adult bones by new cells (osteoblasts). This is the phenomenon of bone turnover (Eriksen 1989, 379; Hedges et al. 2007, 814-815; Hill 1998; Parfitt 1983). Different bones are remodeling in different rhythms; thus, it takes a different amount of years for the collagen carbon to be replaced. For instance, femoral remodeling continues for approximately 25 years, while the ribs turnover every 6 years. Bones such as those in the cranium can reach turnover rates of 50 years, which makes them useful for interpretations in older skeletons (Hedges et al. 2007, 815; Mulhern and Van Gerven 2000, 525). Similarly, mineralization of the bone happens at different rates for different types. For example, cortical bone (which is the dense outer hard part of the bone, also called compact bone) remodels more slowly than trabecular bone (the inner part of the bones, also called cancellous or spongy bone) which has more rapid remodeling rates (Figure 2) (Eriksen 1989, 393-395; Mulhern and Van Gerven 1997, 135,145; Parfitt 1983). The phenomenon of bone turnover should be

taken under consideration when picking the sample type, since it can affect the kind of derived data after analysis.

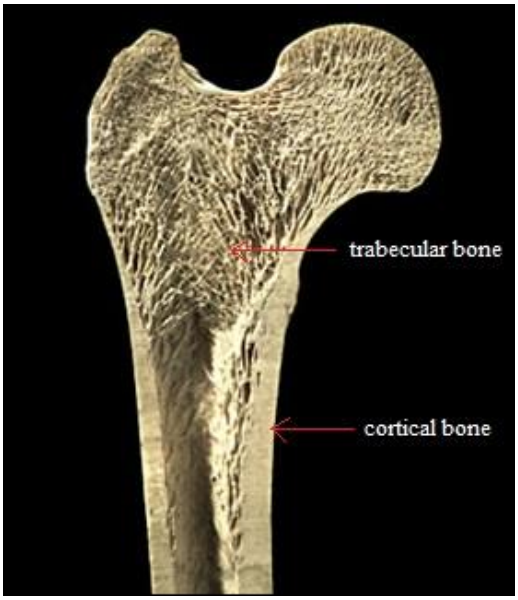



Figure 2 – Bone anatomy. Photo by Paul Crompton, ©University of Wales College of Medicine (www.depts.washington.edu)

There are three kinds of material that can be used as a sample from bones: bone collagen which is organic, or bone carbonate ($CO_3$) and bone phosphate ($PO_4$) which are inorganic (Katzenberg 2008, 416-417).

### *2.1.6.2 Teeth*

Two other significant calcified tissues used widely in archaeological stable isotope analysis come from teeth: the dental enamel and dentine. These two kinds of tissues together with cementum constitute the components of the dentition (Hillson 1996, 8-12) (Figure 3).

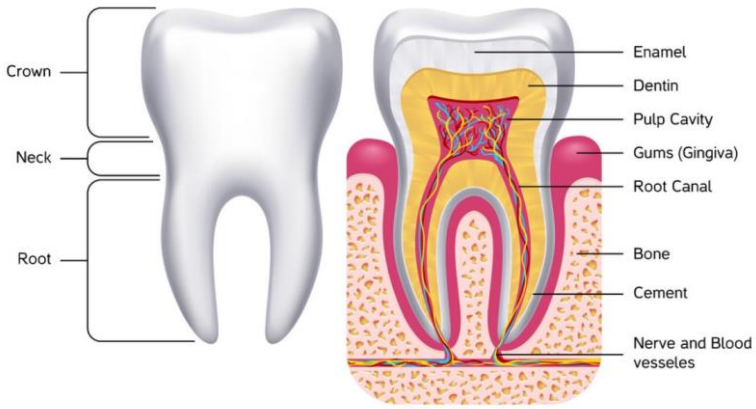



Figure 3 – Anatomy of a tooth. Figure taken from https://www.vectorstock.com/royalty-free-vector/tooth-anatomy-dental-infographics-vector-11229680

Both organic and inorganic components of the dental tissue can also be utilized in isotope analysis. Dentine is the organic part of the tooth and can be used as collagen source, with only 18% less collagen than bone. Dentine is also a source of $PO_4$ (phosphate) components, which can also be found in enamel. Enamel is the inorganic part of the tooth, consisting of more than 99% of well-crystallized hydroxyapatite. This material can be used as a carbonate ($CO_3$) or phosphate ($PO_4$) source in isotope analysis (Hillson, 2005, 146-190; Lee-Thorp 2008, 926; Tsutaya and Yoneda 2015, 7).

Teeth develop during childhood, and the adult teeth are not subjected to turnover and remodeling, unlike bone (Hillson 2005, 152). The different layers form during infancy and childhood and retain their chemical composition after the tooth is completely developed (Hillson 1996, 119; Katzenberg 2008, 416). The formation rhythms have been thoroughly studied through the years and currently display well-defined patterns of development that allow more specific investigations (AlQahtani et al. 2010; Ubelaker 1989) (Figure 4). This way the chemical composition of the tooth seals the information for specific and relatively short periods of the individual's life, providing an unaltered image of the individual's childhood. Hence, teeth can be used as a valuable source of evidence and give insights for the provenance or the diet during childhood (Britton et al. 2015; Richards et al. 2002; Roberts et al. 1988; Wright and Schwarcz 1998, 1999).

Furthermore, enamel shows the best preservation rates among hard tissues, often almost identical to fresh enamel (Hillson 2005, 158). There are also studies that have shown that there are processes that under certain conditions can enhance its preservation (Antoine et al. 1999).

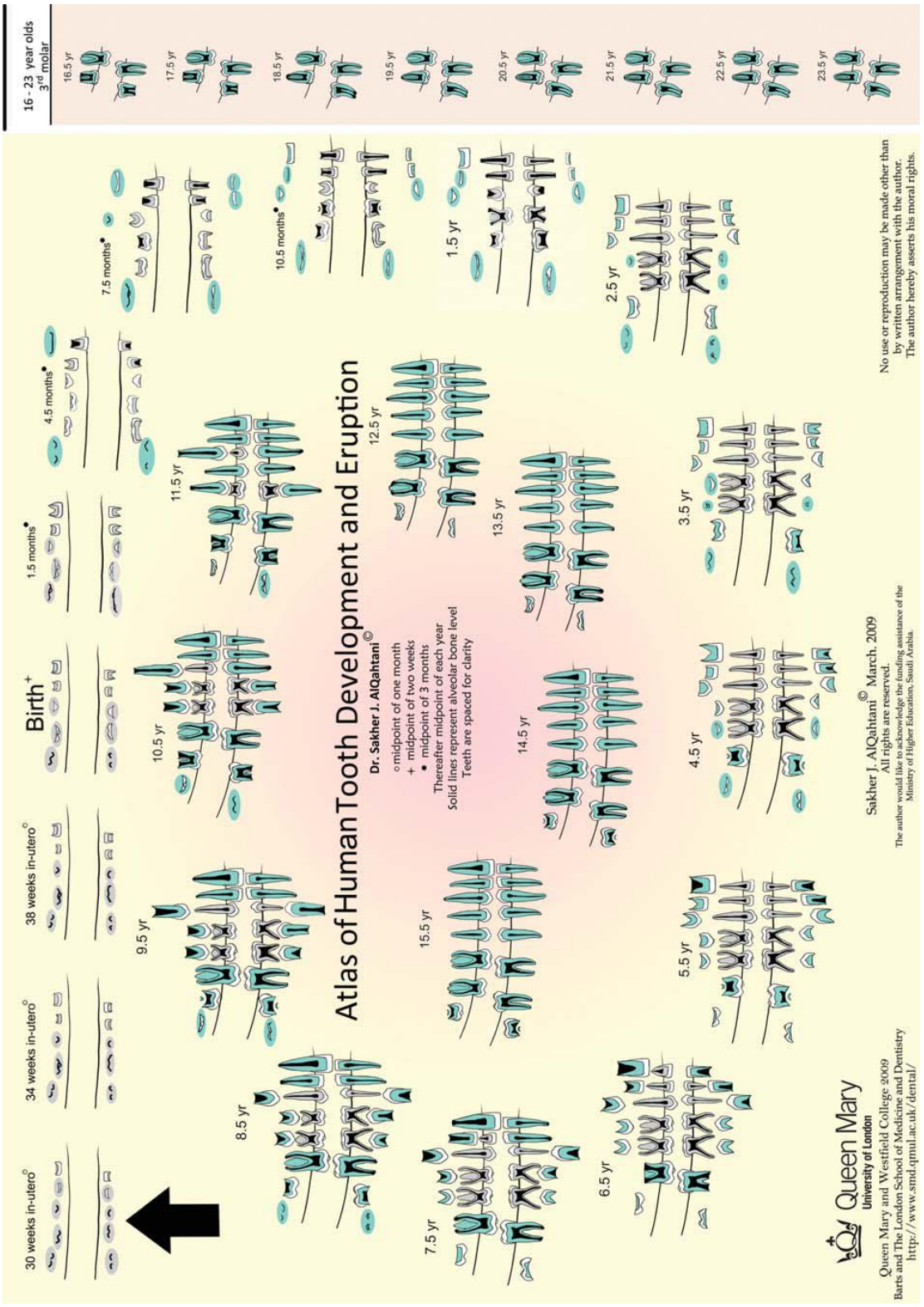



Figure 4 – The development and eruption of human teeth, with the arrow indicating the starting point. The enamel is indicated by white colour and dentine with gray for deciduous and green for permanent teeth (after AlQahtani et al. 2010).

#### *2.1.6.3 Collagen*

Collagen is a structural protein that can be preserved in archaeological remains, found in both the bones and the cementum/dentine of the teeth. In non-skeletal tissues, it can be also found in tendons, skin, muscles and cartilage. Like all proteins, collagen consists of amino acids. The 20 amino acids of collagen are bound together into a triple-helix (Hillson 2005, 148). Collagen is produced in the body using amino acids from food. In isotope analysis, when collagen is used as a sample source, the signatures reflect the isotopic composition of the food consumed, since the latter is retained even after collagen's formation (Ambrose and Norr 1993; Katzenberg 2008, 417). For this reason, collagen has been used widely in paleodietary studies, utilizing nitrogen and carbon isotope ratios which constitute circa 11-16% and 35% of collagen respectively (Katzenberg 2008, 416). It has also been shown that collagen isotopic values reflect mainly the protein that has been ingested during consumption, rather an image of the whole diet (Sullivan and Krueger 1981). Ambrose and colleagues (1990) provided a standard protocol in collagen isolation from bones.

Collagen is very often well-preserved in archaeological remains since is a very stable protein that does not dissolve in water and is fairly resistant to bacteria and fungi (Hedges and Wallace 1978). Diagenesis (or diagenetic contamination) is the degradation of the protein that occurs postmortem and has been studied thoroughly together with its effect on isotopic ratios (Collins et al. 2002, 389-390; DeNiro 1985, 808; Hedges 2002). Collagen progressively degrades at varying rates, with the environment of the burial and the climate playing a great role on its preservation (Katzenberg 2008, 416).

#### *2.1.6.4 Apatite*

The mineral part of the calcified tissues is the apatite and it mainly consists of calcium phosphate. The chemical formula of apatite is $Ca_{10}(PO_4)_6X_2$. It is based on calcium ($Ca^{2+}$) and phosphate ($PO_4^{2-}$) with X usually being either a hydroxyl ($OH^-$) (hydroxyphosphate) or fluoride ($F^-$). Substitutions can take place which can include carbonates ($CO_3$) in the place of $PO_4$ or OH. Either $CO_3$ (carbonate) or $PO_4$ (phosphate) can be used in oxygen isotope analysis of mineralized tissues, where a linear correlation has been observed. Carbonates can be used in dietary interpretations utilizing carbon isotopes. In contrast with collagen carbon, the whole diet is represented since carbonates have their origins in lipids and proteins that have been dissolved in blood (Hillson 2005, 146; Katzenberg 2008, 416-417).

One main disadvantage of apatite as a sample is that it is also susceptible to diagenesis, with apatite isolated from tooth enamel being more preferable than bone (Kohn et al. 1999, 2745). Moreover, diagenesis can also affect $PO_4$ in oxygen isotope analysis although to a lesser degree (compared to carbonate) due to the strength of the P-O bond (Kohn et al. 1999, 2738). There are also concerns expressed regarding postmortem alterations and elemental substitutions that can influence isotopic ratios (Katzenberg 2008, 416-417). To address these issues, researchers developed pretreatment methods which would potentially remove soluble (most possibly diagenetic) carbonates from the sample (Lee-Thorp 1989). Lastly, the advantage of apatite is that it can be used in cases where it is not possible for collagen to be isolated or where is has been badly preserved (Lee-Thorp 1989; Sullivan and Krueger 1981).

## 2.2 Literature record of weaning estimations using isotope analysis

When studying breastfeeding and weaning practices in the archaeological record, one of the main goals is to pinpoint the age of weaning. The timing of BWPs can have implications on population demographics, morbidity and subsistence practices, and even human evolution. Therefore, understanding these processes and refining the time frame and the patterns in which the weaning process occurred in different past societies provides valuable evidence in terms of population dynamics and the archaeology of gender (Jay 2009, 163-165; Mays et al. 2002, 654).

Through the years, the study of breastfeeding and weaning behavior has evolved in a great degree. The majority of the first stable isotope studies on paleopopulations, focusing on BWPs, most used the analysis of nitrogen isotopic values in bone collagen (Fogel et al. 1989; Katzenberg and Pfeiffer 1995; Schurr 1997,1998). Using the isotopic signatures of nitrogen made it possible to track the proteins in the infants food revealing the point where breast milk was no longer ingested. Later on, the additional employment of other chemical elements took place aiming to a more comprehensive picture of the BWPs. Nitrogen signatures were combined with carbon isotopic values providing further insights for the timing of initial supplementation (Dupras 2001; Katzenberg et al. 1993; Tuross and Fogel 1994; White and Schwarcz 1994). In other words, while nitrogen and oxygen can be used to identify the entire period of breastfeeding, carbon may allow the identification of the point when supplementary food is introduced to alongside breast milk (Dupras 2001; Fuller et al. 2006a,b). Teeth samples were also utilized in order to increase the precision of the interpretations, mostly regarding weaning chronologies (Herring at al. 1998; Mays et al. 2002; Richards et al. 2002). After the first assumption that oxygen can contribute in tracking breastfeeding and weaning practices (Roberts et al. 1988) the carbon/oxygen combination on teeth samples started to be employed more in this kind of studies (Wright and Schwarcz 1998,1999; White et al. 2004).

Until present, nitrogen isotopic signatures are still the most frequently used for identification of breastfeeding and weaning practices, usually combined with carbon on organic samples from bone (Bocherens et al. 2007; Burt 2013; Chinique de Armas et al. 2017; Chinique de Armas and Pestle 2018; Clayton et al. 2006;

Howcroft et al. 2012; Jay et al. 2008; Pearson et al. 2010; Redfern et al. 2012). However, there are many different approaches followed within the field, with studies that utilize carbon and nitrogen isotopic values on intra-tooth samples (Beaumont et al. 2012; Sandberg et al. 2014). Others use a combination of carbon and oxygen isotopic signatures on teeth (Dupras and Tocheri 2007) or all three elements on bone samples (Britton et al. 2015; Williams et al. 2005).

The conclusions drawn regarding the age of weaning in these studies are relatively straightforward, but cannot be fully precise. Conclusions are most commonly phrased according to observations of isotopic change that signifies exclusive breastfeeding, cessation of breastfeeding or supplementation. Therefore, these conclusions usually include the age by which the children no longer receive breast milk (Bocerens et al. 2007; Richards et al. 2002), the timing where isotopic values of plant or animal food supplements are starting to be observed (Fuller et al. 2006; Jay et al. 2008) or the minimum age that the infants were still being breastfed (Clayton et al. 2006). In human populations the estimation of the age of weaning falls mostly around or before the age of two, with the introduction of liquid or solid food (supplementation) suggested at around six months of age (Izurieta and Larson-Brown 1995; Sellen 2001; Wright et al. 2004). This age is regarded as the healthiest point of an infant's life to stop receiving exclusively breastmilk (WHO 2002). Although it has been suggested that supplementation before the age of six months can increase morbidity, it can be used as an extra resource of energy for an already sick infant (Wright et al. 2004).

## 2.3 Relative studies in the Caribbean

The application of isotope analysis has played an important role in the study of diet and mobility of the Caribbean region. Together with the study of material culture, isotope analysis has provided extra insights on tracking the first inhabitation of the islands, the provenance of the people, their dietary habits and interpreting signs of early domestication. For instance, as mentioned in section 3.1.1, the Archaic period was characterized as preceramic and preagricultural. Although the presence of clay artifacts can be observed macroscopically, paleobotanic evidence may not survive due to taphonomical factors. Isotope analysis can facilitate this problem through dietary analysis of the skeletal remains indicating the consumption of agricultural plants, which would suggest cultivation practices ($C_4$ plants, see section 2.1.5.1) (Buhay et al. 2013; Chinique de Armas et al. 2015). Furthermore, it has been shown that strontium and oxygen analysis can be utilized to indicate nonlocal people (Laffoon et al. 2013). Also, the predominance of subadult skeletal remains in northwestern pre-colonial sites of Cuba provides valuable samples for the study of pre-weaning and post-weaning periods (Chinique de Armas 2017, 2018; Reid 2018, 103). More specific evidence of the application of isotope analysis in the Caribbean will further be examined in the next paragraphs, focusing on dietary studies and breastfeeding practices.

### 2.3.1 Dietary studies

Paleodiet is the term that is used to describe the diet reconstruction of early humans. It can employ cross-disciplinary analytical approaches and it is based on the types of food presumed to have been consumed by individuals in the past. The variation in carbon and nitrogen isotopic values are used in many recent studies to predict the characteristics of paleodiet, and constitutes one of the most essential methods to study ancient subsistence regimes or subsistence economies (Pestle and Ramos, 2018, 272; Smith et al. 2018, 98). After isolation and purification of the tissue under study (either collagen or apatite) the comparison of $\delta^{13}C$ and $\delta^{15}N$ ratios can give estimates on the dietary basis of the individuals. These estimates regard the contribution of each food group to the diet of the population and can be meaningful in cases where there are distinct differences among the food classes

consumed, the tissue is well-preserved, and its fractionation is well understood (Ambrose and Norr 1993; Lee-Thorp 2008; Pestle and Ramos 2018).

In the case of the Caribbean, the application of isotope analysis has given valuable insights regarding the introduction of cultivation and agriculture, tracing the shift from resource extractors to resource producers and therefore, farmers (Pestle and Ramos 2018, 274; Smith et al. 2018, 99). The oldest evidence of plant domestication in the Caribbean at present comes from Trinidad, around 4600 B.C., where indications of maize were found (Pagan Jimenez et al. 2015).

In recent years, Cuban archaeological sites in the western part of the island have provided important evidence of complexity in subsistence practices on the island during the Archaic period, also in comparison with other neighboring islands of the Greater Antilles (Ruiz and Beatón 2000, 1; Chinique de Armas et al. 2017; Smith et al. 2018, 98). More specifically, four archaeological sites in western Cuba were selected from Chinique de Armas and colleagues for comparison of their dietary basis. Based on radiocarbon dating results the sites Canimar Abajo, Guayabo Blanco, Cueva del Perico I and Cueva Calero believed to have been contemporaneous, which allowed the assessment. In their first article in 2015, following macroscopic indications of starch presence in the diet of Canimar Abajo population, they found evidence of 79 starch granules present on the dental calculus of six individuals (Chinique de Armas et al. 2015). The evidence suggested early cultivation practices taking place in the Late Archaic period which was a unique characteristic of Canimar Abajo in comparison with the other three sites. In a follow up study from 2016, Chinique de Armas and colleagues found the diet in Canimar Abajo to be closely related with sites in other islands in the Greater Antilles, which had already been characterized as agricultural in the literature (Buhay et al. 2013; Chinique de Armas et al. 2017).

Isotope dietary studies such as those performed on the Cuban populations have also been conducted for other islands in the Caribbean, comparing carbon and nitrogen isotopic signatures of populations from different sites, indicating the complexity of the subsistence regime of the early settlers and their food network (Laffoon and de Vos 2011; Laffoon et al. 2013; Norr 2002; Pestle 2010; Schroeder et al. 2009; Smith et al. 2018, 108; Wright and Schwarcz 1998).

### 2.3.2 Breastfeeding and weaning practices

It has only been in the last decade that the study of BPWs has been developing in the Caribbean. Although, a lot of research studies have been conducted on general dietary reconstructions of adults, there are only a few that focus specifically on how breastfeeding and weaning practices were affected by different subsistence economies using isotope analysis (Buhay et al. 2013; Chinique de Armas et al. 2017; Chinique de Armas and Pestle 2018; Pestle 2010).

Buhay and colleagues (Buhay et al. 2013) conducted an isotopic investigation of 28 individuals from Canimar Abajo site in Cuba. The study included carbon and nitrogen isotope analysis of bone collagen samples from seven subadults that fell in the category of infants/juveniles (age range of 10-22 months, n=7). After the comparison of the subadult isotopic values with female adult isotopic signatures within the population, they observed that some or most of the infants/juveniles were weaned with the usage of terrestrial plants as supplementation food. However, no specific estimation of the age of weaning was ascertained with certainty.

Four years later, a second study of individuals from the same site was published by Chinique de Armas and colleagues (Chinique de Armas et al. 2017) using Bayesian probability models for their interpretations to fill the gaps of the previous study. Carbon and nitrogen isotopic analysis of bone collagen were analysed for 31 juveniles and 18 female adults from the Canimar Abajo population of fisher-gatherers/horticulturalists. Their results provided indications that could be used to obtain a weaning age estimation. Their conclusion suggested that up to two years of age infants were still breastfeeding with some supplementation food ($C_3$ plant based), which resulted in complete weaning by the age of three. This estimation agrees with precedent ethnographic (Maybury-Lewis 1974,73) and isotopic (Pestle 2010, 319) studies from the general region of the Caribbean, focused in the area of Puerto Rico, and give an estimation of weaning age at around 2-3 years of age.

One of the latest studies is that of Chinique de Armas and Pestle (2018), who conducted a broader geographically isotopic BWPs study which focused on six precolonial populations of three different islands (Cuba, Puerto Rico and Belize). Different subsistence economies had already been assigned to these populations, and the researchers' aim was to distinguish differences in BPWs

between them. Utilizing stable carbon and nitrogen isotope analysis in bone collagen and apatite, they discovered variation of weaning age between groups that had different subsistence economies. More specifically, they propose that weaning was starting in an earlier age for hunter-fisher-gatherers than agricultural populations, in contrast with the prevailing paradigm in biological anthropology. Furthermore, they examined the variation of the food used for children's weaning in the nitrogen isotopic values, which indicated that this choice was directly correlated with the food and cultigens availability (Chinique de Armas and Pestle 2018).

Isotopic analysis studies on breastfeeding and weaning practices in the region of the Caribbean have focused mostly on stable carbon and nitrogen isotope analysis. Moreover, these studies primarily use bone samples. As discussed in this chapter (section 2.1.6.3), bone collagen is not only more susceptible to diagenesis, but the bone itself also remodels and does not always allow specific chronological interpretations. Conversely, the study of deciduous and permanent teeth can be highly informative, pinpointing specific time periods and patterns of breastfeeding and weaning practices in paleopopulations. Furthermore, there are currently no studies utilizing the combination of carbon/oxygen isotopic signatures that focus in BWPs, although the usage of oxygen on teeth samples has been studied in other populations leading to valuable new evidence (Britton et al. 2015; Dupras and Tocheri 2007; Wright and Schwarcz 1998,1999).

Considering prior studies, the present study intends to contribute to the field by utilizing an alternative approach to provide new evidence in the study of BWPs in the region of the Caribbean, and more specifically, in Archaic Cuba.

# 3 MATERIALS AND METHODS

## 3.1 Contextual Background

### 3.1.1 The Archaic Period of Cuba

The earliest Caribbean people arrived in Trinidad in 5500 B.C. one of the islands opposite to the South American mainland (Venezuela's coasts today) during what is known today as the Archaic Period of the Caribbean (Reid 2018, 9). The island complex of the Caribbean was the last large uninhabited part of the Americas to be populated (Saunders 2005, 84; Wilson 2007, 1). The first people who arrived were hunters-fishers-gatherers. The Archaic era is characterized by an abunadance of stone, shell and bone tools, with little pottery production or agriculture (Reid 2018, 9-10). However, due to the fact that there were cases where pottery was found to be included in Archaic archaeological findings (Richie 1965, 31-32) or there were indications of "early experimentation in plant domestication" (Buhay et al. 2013; Chinique de Armas et al. 2015, 2016; Willey and Phillips 1958, 107), the Archaic Period in the Caribbean complex is regarded as a developmental stage (considered as preceramic and preagricultural), a period where people were trying to adapt in novel environments. The Ceramic Age followed the Archaic period and the shift took place gradually once horticulture and pottery production became fully developed around 800-200B.C. (Reid 2018, 12-13).

The first people are believed to have arrived in most of the islands from South America and Mesoamerica between 5000-4000B.C. (Saunders 2005, ix) (Figure 5). Stone tools that were found in Archaic archaeological sites show great similarity with tools found in Yucatan peninsula supporting the idea that there were also people who migrated from Central America (Wilson 2007, 4). On the other hand, there are some archaeologists who found the tools to be similar to traditional stone technology from North America, suggesting migration of people from modern dayFlorida and the Bahamas (Saunders 2005, xii). By contrast, Granberry suggested migration towards Florida occurred from northwestern Amazonia and through the Caribbean (Granberry 1993, 41).

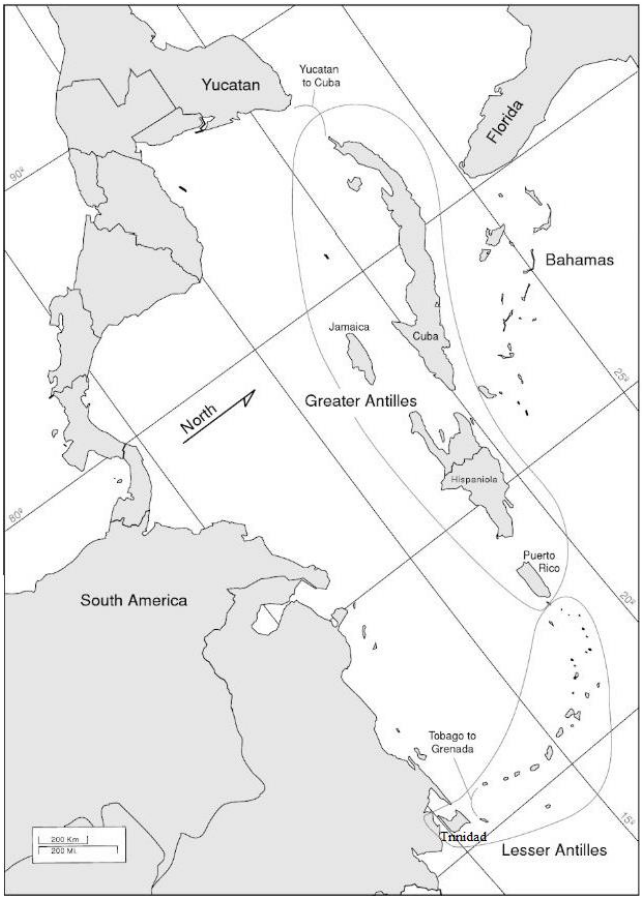



Figure 5 – Map of the Caribbean and the closer parts of mainland Americas (after Wilson 2007)

North Caribbean islands (Greater Antilles) show signs of human habitation in a later time (Saunders 2005, xii). Cuba is the largest of the Caribbean Islands (Saunders 2005, 84) where Casimiroid people were the first settlers around 4000 B.C. Archaeological evidence from the Levisa rock shelter suggests that it is possibly the oldest rock shelter of the island, dating to about 4190 B.C. Aside from rock shelters, many ancient sites in Cuba are in caves, located along the coast or at the inner valleys of the island. Archaic period sites in Cuba include workshops for stone tools, like blades and hammer stones as well as bone and shell artifacts (Saunders 2005, 84-85; Smith et al. 2018, 99).

### 3.1.2 The site

During the early 20th century, discoveries of funerary sites were not that common in the western region of Cuba. It has only been in recent years that this region became one of the most notable ones for the study of ancient skeletal remains after archaeological discoveries in several important sites in recent years (Ruiz and Beatón 2000, 1; Garcell Dominguez 2006; Roksandic et al. 2015).

Cueva del Infierno (or also Sitio Bacuranao I) is a cave located in San José de las Lajas, in the Mayabeque province of Havana, at the northwestern part of Cuba in the Caribbean (Figure 6). The site was excavated in 1995 and literature about the excavation is limited to one archaeological report (Garcell Dominguez 2006) and one anthropological report (Ruiz and Beatón 2000). The excavation was led by the Anthropological Center of CITMA (Ministry of Science, Technology and Environment) and the Speleological Society of Cuba (Sociedad Espeleológica de Cuba (SEC) (Garcell Dominguez 2006, 64-65).


Figure 6 – Location of the Cueva del Infierno site (yellow star sign), northwestern Cuba, Caribbean. Figure generated by the author using Map data ©2019 Google, INEGI.

The cave was found to be an important funerary site, which included two cemeteries, Cemetery I and Cemetery II, with mostly primary burials. Cemetery I was 22 $m^2$ with a general concave and irregular profile, running from the edge of

the roof to the bottom of the cave. Cemetery II was more related to an area of activity or a room, and had a conical shape (Garcell Dominguez 2006, 72-74).

Two charcoal samples were used for radiocarbon analysis. The dating was carried out in the laboratories of the Wissenchaften Academy in Heidelberger, Germany, giving dates that placed the population with the first settlers in the region. Cemetery I was dated at 1625-1525 B.C. and Cemetery II at 1425-1400 B.C. According to the data, the population was chronologically assigned to the pre-colonial era of Cuba (Archaic Period) (Garcell Dominguez 2006, 64-65).

### 3.1.3 The osteological study

The anthropological study was conducted by Rafael Travieso Ruiz and Lisette García Beatón in 1997 at University of Habana, faculty of Biology. The excavation of the two cemeteries recovered an assemblage which constituted of skeletal remains that were initially grouped into 54 human burials. After the osteological study was completed, the minimum number of individuals was raised to 66 and included three fetuses, 52 infants, two adolescents and nine adult burials (Table 1). A part of the sample was found painted red, likely using hematite tincture stone which insinuates a funerary ritual for populations like the one under study (Ruiz and Beatón 2000, 2-3).

Table 1 – List with the age categories of the individuals of the human burials (translated from the author after Ruiz and Beatón 2000)

| ***Age category*** | ***Number of burials*** | ***Percentage (%)*** |
|---|---|---|
| *Fetus (< newborn)* | 3 | 4.5 |
| *Infant (0-3 years)* | 52 | 78.7 |
| *Child (3-12 years)* | 0 | 0 |
| *Adolescent (12-20 years)* | 2 | 3 |
| *Adult (>20 years)* | 9 | 13.6 |
| *Total* | 66 | 100 |

For the majority of the individuals analysed, the osseous parts which are normally used for an accurate sex estimation (skull, long bones and pelvis) were absent or poorly preserved. From the nine adults, only one had diagnostic parts available and after the analysis of its skull and long bones, the individual was

estimated as a female. As for the case of the subadults, sex estimation was not conducted due to the lack of studies that can provide accurate sex estimation for children's remains, since in that age categories there is only little, or no development of sexual characteristics expressed in bone tissue (Ruiz and Beatón 2000, 2).

Although there were not a lot of bones showing pathological signs, inflammatory processes of the long bones appeared to be the most common pathological indicator. Other observations included “periosteal reaction, osteolytic damage in the form of perforations, congenital deformation of the occipital hole and osteoarthritis” (Ruiz and Beatón 2000, 3).

## 3.2 Sampling strategy

Two objectives were taken under consideration before the setup of the sampling strategy. The first one is the acquisition of an overall image of the people of Cueva del Inferierno by looking at their entire diet through carbon isotopic analysis (DeNiro and Epstein 1978; Ambrose and Norr 1993) and searching for provenance using oxygen isotopic ranges (Chenery et al. 2010; Laffoon et al. 2013; Schroeder et al. 2009). The newly generated data will place the population in the broader Caribbean by comparing the results with recent findings from other populations of the same island (Chinique de Armas et al. 2015, 4; Laffoon et al. 2013, 7; Smith et al. 2018, 102-105). The second and main purpose of this study is to derive information about changes in the isotopic signatures of the chemical elements during specific periods of the individual’s life and more specifically, trace patterns of variation attributable to BWPs. For this reason, a time range is desired to include evidence of isotopic ratios before and after birth, which will allow observations of the isotopic fluctuations of carbon and oxygen during breastfeeding and early supplementation.

Considering the objectives of the study, a decision was made regarding the nature of the samples. For this study, teeth were selected instead of bones for two reasons; firstly, it is known that human enamel is less susceptible to diagenesis compared to bone (Kohn et al. 1999, 2738) and secondly, the development of teeth is occurring in certain periods of one’s lifetime. These developmental patterns and rates have been studied in depth and although there is slight inter- and intra-population variations, they are still considered to be a great source of evidence when

specific time periods of life are targeted (AlQahtani et al. 2010, 485; Ubelaker 1989, 64).

With the above considerations in mind, the first step was to sample all individuals, selecting one tooth, aiming preferably for the first permanent molar ($M_1$). For the second part, which includes sampling for specific time periods in life, the developmental stages and the timing of the enamel deposition on the teeth were taken under consideration (AlQahtani et al. 2010, 485). Keeping in mind the developmental rate varies amongst populations (Ubelaker 1989, 69) and in order to gain more insights of the patterns of isotopic fluctuations, the sampling strategy focused on three different teeth types that would represent three different but important periods of development: the time *in utero*, the time around birth, and the time directly after birth. The three types of teeth selected were: One deciduous incisor (dI) that represents the time *in utero*, one deciduous molar (dM) that reflects the time period around birth and one permanent first molar ($M_1$) which develops in the first year of life and the enamel deposition continues up to the third, providing information about the beginning of the weaning process (Figure 7). The nomenclature used for the dental identification is presented in (Figure 8). The primary and most important goal of this part of the sampling strategy is to isolate multiple samples from different teeth from the same individual. This way, the results will depict the processes occurring within the same person rather than attempting to correlate observations for different periods of life of different individuals.

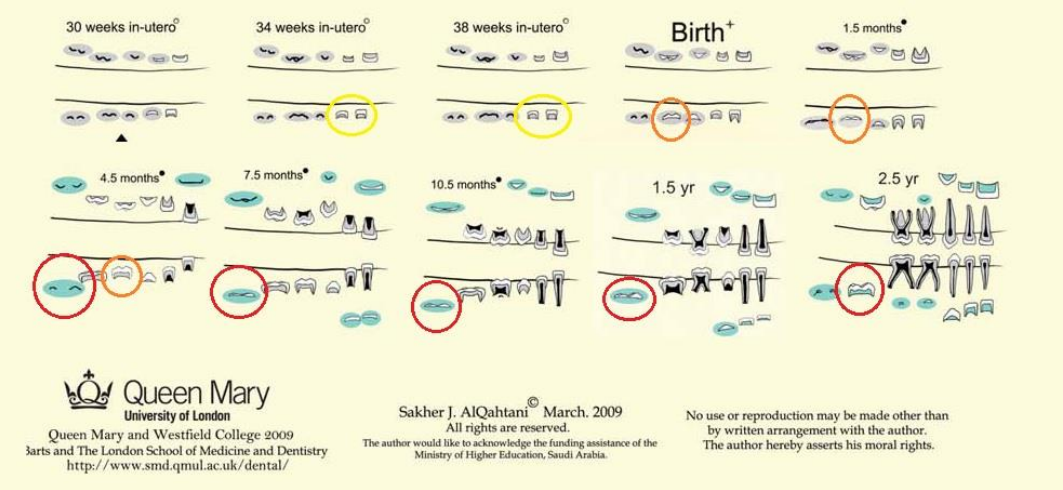



Figure 7 - The development and eruption of the teeth, after AlQahtani et al. 2010. Deciduous incisors are circled with yellow, deciduous molars with orange and first permanent mandibular molars with red. The ages depicted in the picture are calibrated by 40 gestational weeks according to O'Neil 2005.

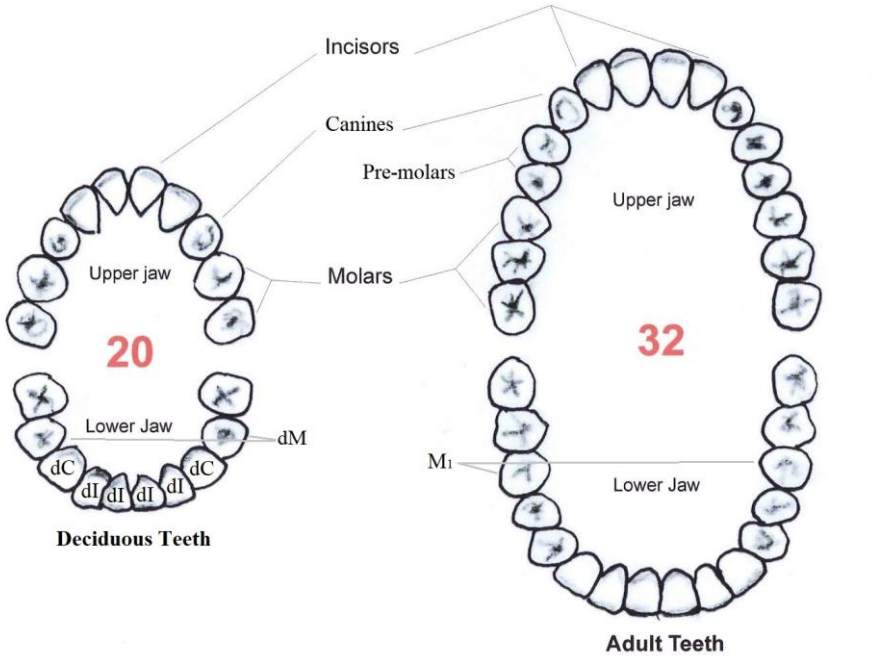



Figure 8 – Nomenclature for the dental identification used in this study

## 3.3 Sampling material

Due to the limited access to the archaeological collection, the selection of the individuals sampled was mostly arbitrary. Forty-nine samples were taken from 34 different individuals of the Cueva del Inferierno population. To serve the purposes of the study, among the sampled teeth were five deciduous incisors (dI), two deciduous canines (dC). In the cases that no deciduous incisor was available, fifteen deciduous first molars (dM), 25 permanent first molars ($M_1$) and two permanent canines (C) when the first permanent molar was not available (data presented in Table *2*). All teeth sampled were extracted from the mandibles. The main goal was to obtain a triplet of samples from as many individuals possible. These triplets include: One deciduous incisor (dI), one deciduous molar (dM) and one permanent first molar ($M_1$). Sampling at least two of the teeth mentioned was possible for four individuals and a triplet of teeth was extracted from five, creating a subgroup of nine individuals that were multi-sampled. The individuals included in this phase and the teeth sampled are presented in bold in Table 2.

Table 2 - List of individuals and the teeth sampled, deciduous incisors (dI), deciduous canines (dC), deciduous first molars (dM), permanent first molars ($M_1$) and permanent canines (C). The individuals that were multi-sampled are in bold.

| *Individual* | *Tooth type* | *Individual* | *Tooth type* | *Individual* | *Tooth type* |
|---|---|---|---|---|---|
| *CI-E1* | C | ***CI-E30 M*** | **$M_1$** | ***CI-E6*** | **dI** |
| *AdultoB* | | ***CI-E30A*** | **dM** | ***CI-E6*** | **$M_1$** |
| *CI-E1C* | dC | *CI-E31* | dM | ***CI-EE*** | **$M_1$** |
| *CI-E1C* | $M_1$ | ***CI-E35*** | **dI** | ***CI-EE*** | **dM** |
| *CI-E1D* | $M_1$ | ***CI-E35*** | **$M_1$** | *CI-I22* | $M_1$ |
| ***CI-E21*** | **dI** | ***CI-E35A*** | **dM** | *CI-I23* | $M_1$ |
| ***CI-E21*** | **$M_1$** | ***CI-E36A*** | **dM** | *CI-I25* | $M_1$ |
| *CI-E21B* | dM | ***CI-E36A*** | **$M_1$** | *CI-I26* | $M_1$ |
| *CI-E21C* | C | ***CI-E39*** | **$M_1$** | *CI-I27* | $M_1$ |
| *CI-E21D* | $M_1$ | ***CI-E39*** | **dM** | *CI-I28* | $M_1$ |
| *CI-E21E* | $M_1$ | *CI-E40A* | dM | *CI-I29* | $M_1$ |
| *CI-E21F* | $M_1$ | ***CI-E49*** | **dI** | *CI-I31* | $M_1$ |
| *CI-E28* | dM | ***CI-E49*** | **dM** | *CI-I40* | $M_1$ |
| ***CI-E29*** | **dC** | ***CI-E49A*** | **$M_1$** | *CI-I41* | $M_1$ |
| ***CI-E29*** | **$M_1$** | *CI-E51A* | dM | *CI-I43* | $M_1$ |
| ***CI-E29A*** | **dM** | *CI-E6* | dM | *CIE34A* | dM |
| | | | | *MII-E21* | dM |

The teeth were generally well preserved. Some of the teeth were very fragile due to their incomplete development. Unfortunately, further information about the individuals of Cueva del Inferierno could not be recovered, since there has been no data published yet for this archaeological collection and the only source available was the Spanish version of the excavation report (Garcell Dominguez 2006) as well as the anthropological report that does not include age estimations (Ruiz and Beatón 2000).

## 3.4 Processing

### 3.4.1 Labelling

Firstly, the samples were placed in 15ml eppendorfs that were assigned with a list number and labeled with the sample number and the tooth type. Some of the samples were already loose teeth but others needed to be extracted from the mandible with the help of pliers.

### 3.4.2 Washing

After the labelling, a step of washing was performed in order to remove any excess soil residues. The procedure started by filling the two thirds (2/3) of the eppendorfs with distilled water ($dH_2O$). Then, the eppendorfs were transferred in the Elmasonic One - Ultrasonic Cleaning Device by Elma Ultrasonic Technology for approximately 60 minutes. The sonicator used ultrasounds (35 kHz) which were transferred in water median to agitate the fluid in the eppendorfs that contained the sample. This way traces of soil or other kind of contamination that were tightly adhered or embedded onto the solid surface of the tooth were thoroughly removed (Dietz and Badavinac 2002, 129). After the sonication treatment the eppendorfs were transferred to a rack after pouring out the water. A pipette with a clean tip was used to speed up the drying process, removing a greater amount of water. The last step was to leave the samples in the fume hood until they were completely dry.

### 3.4.3 Drilling

After the samples were completely dry the drilling of the enamel powder was performed with a Proxxon hand-held diamond-tipped drill. Before the drilling of each tooth the diamond burr was cleaned by submerging it multiple times in three different solutions: Ethanol (EtOH) 95% – to sterilize the end of the drill-, hydrochloric acid (HCl) with concentration 0.6M – to dissolve inorganic material on the diamond drill end that may occur after drilling a tooth to avoid cross-contamination before drilling the next one- and distilled water ($dH_2O$) – to clean the end of the drill from the solutions above.

One tooth was taken out every time and placed on weighing paper to avoid contamination and to ease handling of the powder afterwards. The drill was firstly used to clean mechanically the tooth removing a thin layer of the surface enamel on the desired sampling area, exposing the white and clean core enamel. Wherever was possible (regarding preservation and development of the tooth under sampling) the drilling was focused on specific layers of the crown in order to obtain samples that would be more precise to a certain time period of the individual's life. As mentioned above, the desired time frames were before, around and after birth. The drilling focused on the upper part of the deciduous molar crown and on the middle-upper part of the permanent molar crown wherever possible.

The aim was to collect at least 0.6mg of enamel powder. The powder was placed in clean 1.5ml eppendorf tubes, which were weighed empty and then weighed again with the enamel powder inside in order to monitor the amount of extracted enamel. Weights were taken with an electronic balance by Kern ABS AND Sohn GmbH.

### 3.4.4 Enamel hydroxyapatite Pretreatment Protocol

In order to minimalize the effects of diagenesis in the resulting isotopic signatures it is important to perform a pretreatment procedure after the dental enamel isolation (Epstein et al. 1953, 1315). This procedure is constituted by two steps, the removal of the organic materials and the removal of labile carbonates (Koch et al. 1997, 419-420). The organic materials can play a significant role in the composition of the released carbon dioxide ($CO_2$) that is measured during mass spectrometry (Epstein et al. 1953, 1317-131). The secondary carbonates that may

occur on the tooth as cement can result in biases when the initial structural carbonate of the tooth is measured (Koch et al. 1997, 418; Krueger, 1991, 357).

There are two common approaches to remove the organic matter from the enamel powder: 1) by soaking it in a solution of sodium hypochlorite (NaOCl) (Schoeninger and DeNiro 1982) or 2) hydrogen peroxide ($H_2O_2$) (O'Neil et al. 1994). Koch et al. in their study in 1997 have shown a shift of the $\delta^{13}C$ signatures resulted after treatment with 2% NaOCl (Koch et al. 1997, 420) in comparison with untreated samples. Furthermore, Grimes and Pellegrini in 2013 have shown that 3% NaOCl is more effective than using $H_2O_2$.

For the removal of nonstructural carbonates, calcium acetate buffered with acetic acid is used. The combination of the solution was introduced by Koch et al. in 1997 after years of debate among various concentrations of different acetic acid solutions (Schoeninger and DeNiro 1982; Krueger, 1991).

In this study, the protocol for the pretreatment of the human dental enamel before the analysis is based on the protocol in Bocherens et al. 2011, which includes the usage of 2.5% NaOCl and 1M calcium acetate buffered acetic acid. The processing protocol was revised once in October 2014 and again here for this study (February 2019) as follows:

#### *3.4.4.1 Removal of the organic material (bleaching step)*

First, a bleach solution of the desired concentration was prepared mixing one part of 5.0% sodium hypochlorite (NaOCl) solution with one part of MilliQ water in order to produce a 2.5% NaOCl (bleach) solution. Then, 1ml of the 2.5% NaOCl solution was added in each eppendorf that contained powdered enamel sample. Vortexing of the eppendorfs followed until the enamel powder was fully immersed in bleach. The samples were left at room temperature for 20-24 hours. The next day, the samples were centrifuged for 4 minutes at 2000 rpm. This step created an enamel precipitate and made the removal of the liquid bleach easier. The bleach solution was carefully removed using a clean pipette with a clean tip. Here, the contact of the pipette tip with the enamel precipitate was avoided. The next step was rinsing, where 1ml of MilliQ $H_2O$ was added in every eppendorf that contained the enamel precipitate. The eppendorfs were vortexed until the white precipitate on the bottom was mixed totally in water. This step brought water in touch with any

remaining bleach attached to the enamel powder. After the precipitate was diluted, the samples were centrifuged for 4 minutes at 2000 rpm in order to create once again a new precipitate that helped the removal of the liquid phase in the eppendorf. Removal of the bleach-water liquid was facilitated by a pipette using a clean tip, again avoiding the contact of the tip with the enamel. The rinsing step was repeated three times in order to remove all traces of bleach from the samples.

#### *3.4.4.2 Removal of secondary carbonates*

After the last rinsing step was performed 500μl of acetic acid-calcium acetate buffer (pH=4.75) of 1M concentration was added in each eppendorf that was fully rinsed from bleach. The samples were left at room temperature for less than one hour in order to avoid possible recrystallization of the carbonates. Centrifuging followed for 4 minutes at 2000 rpm. As above, this step created an enamel precipitate. The solution was removed with a clean pipette using a clean tip and avoiding the contact of the tip with the enamel precipitate. The next step was to rinse again to clean the samples from the acetic acid-calcium acetate buffer. As was previously performed, 1ml of MilliQ $H_2O$ was added to every eppendorf that contained enamel precipitate. The samples were vortexed until the white precipitate on the bottom was totally diluted in water. This step brought water in touch with any remaining acetate attached to the enamel powder. Then the samples were centrifuged for 4 minutes at 2000 rpm in order to create once again a precipitate that would accommodate the removal of the liquid phase in the eppendorf. The acetate-water liquid was removed with a pipette using a clean tip avoiding contact with the enamel. This rinsing step was repeated three times in order to remove all traces of acetic acid-calcium acetate from the samples.

### 3.4.5 Drying and transfer

After the last rinsing step, the samples were placed in the fume hood for approximately 48 hours to dry with their cap open and the hood front cover closed to limit sample exposure to contamination. When the samples were completely dry, *ca.* 0.75mg of enamel was transferred to a labelled glass vial of large (L) size and the cap was firmly attached on it. The samples were transferred to the Faculty of

Earth and Life Sciences, at Amsterdam's Free University (Vrije Universiteit Amsterdam -VU Amsterdam) for analysis.

## 3.5 Isotope Analysis

The analysis of the samples took place at VU Amsterdam. A Finnigan DELTAplus Isotope Ratio Mass Spectrometer was used to measure the oxygen and carbon isotopic values. The mass spectrometer uses a reaction of orthophosphoric acid (H3PO4)[100%] with the enamel in the sample, where the acid breaks carbonate bonds releasing $CO_2$, which is what the spectrometer measures on a Gasbench II universal automated interface. The $\delta^{13}C$ and $\delta^{18}O$ values measured provided extra information about the mean values and standard deviations of each measurement. The results were calibrated using the Vienna Pee Dee Belemnite (VPDB) standard with the international reference material IAEA-603 with a standard deviation of 0.1‰ for carbon ratios ($\delta^{13}C$) and <0.2‰, for oxygen isotopic signatures ($\delta^{18}O$).

## 3.6 Statistical analysis

It is important to statistically examine whether or not the isotopic variances derived from the isotope analysis correspond to a statistically significant difference. Descriptive statistics describes the data present but also introduces limitations when further generalizations about the data are attempted. Inferential statistics helps to generalize the findings and allows inferences to be made about samples we do not have, for example; information about the population beyond the description of the present sample. Therefore, it is important to examine if that difference is reliable.

A t-test checks if the means of two groups are reliably different and is based on the following equation:

$$t = \frac{\text{variance between groups}}{\text{variance within groups}} \qquad (2)$$

The wider and more scattered the scores within the groups compared, the more difficult it is for a statistically significant difference to be detected. In other words,

a t-test gives the signal (difference) of the noise ratio in the sample, the less the noise the easier to detect the signal. The p-value corresponding to the resulting t-value expresses the likelihood of the presence of a real difference, namely the pattern produced from the data can be produced also from random data. In order to characterize an observed difference as statistically significant, the p-value should be less than 0.05 ($p<0.05$), which indicates that there is less than a 5% chance that the data can be produced by random data (Field 2009).

For the present sample a paired-samples (otherwise dependent-samples) t-test was used to explore significant differences in the mean $\delta^{18}O$ values between the time before and after birth. The dependent t-test is applied when measurements of the same group of individuals are taken in two different times and each score is paired with another one that was taken from the same individual in a different time period.

# 4 RESULTS

In this chapter the results of stable carbon and oxygen analysis will be presented, together with all the tables and graphs created to help with observations and further interpretation. The dental enamel isotopic values of all 34 individuals, for both carbon and oxygen are shown in Table 3, with ratios reported in the δ notation in parts per thousand (‰). The table provides the mean isotopic values and the standard deviation (SD or σ) of both chemical elements. The mean value of $\delta^{13}C$ ratios is -12.4 ± 0.7 ‰ (1σ) with the maximum value at -10.6‰ and the minimum value at -13.7‰. The mean $\delta^{18}O$ value is -3.3 ± 1.1 ‰ (1σ) with the ratios ranging from -1.1‰ to -6.4‰. The values marked with blue in this table had a lower signal intensity due to the sample quantity and elemental concentrations of the enamel extracted. In such cases, the signal of these samples was weaker and thus they have a higher measurement error. Although they have a relatively higher standard deviation than the rest samples, the isotopic signature itself is not believed to be altered in a great degree and the ratios still fall within the expected ranges. Therefore, the error is assumed to be of a minimum impact on the results. All above considered, the results are regarded reliable for further analysis.

## 4.1 Isotope analysis for both carbon and oxygen

### 4.1.1 Overall data analysis

Firstly, the ratios for both carbon and oxygen signatures are plotted together in a scatter plot with $\delta^{18}O$ (‰ vs VPDB) values on the y-axis and $\delta^{13}C$ (‰ vs VPDB) values on the x-axis (Figure 9). The values in this graph represent all 34 individuals that were sampled, divided into three groups; deciduous incisors (including deciduous canines) (dI), deciduous molars (dM) and permanent first molars (including a few permanent canines) ($M_1$). The deciduous and permanent canines were grouped together with deciduous incisors and permanent molars respectively. Given that the deposition of the enamel of the teeth in the same group occurs around the same age, they are expected to give information for approximately the same life period of the individual (AlQahtani et al. 2010, 485).

Table 3– Results of the carbon and oxygen isotope analysis of human dental enamel provided by VU Amsterdam. The table presents the individual, the tooth type [deciduous incisor (dI), deciduous canine (dC), deciduous first molar(dM), permanent canine (C) and permanent first molar ($M_1$)], the mean values (average), and standard deviation (SD) of $\delta^{13}C$ and $\delta^{18}O$ measured in parts per thousand (‰) and the strength of the analytical signal [peak I(m/z 44) intensity]. Samples with lower signal intensity are marked with blue.

| | | *$\delta^{13}C$ (‰ vs VPDB)* | | *$\delta^{18}O$ (‰ vs VPDB)* | | |
|---|---|---|---|---|---|---|
| *Individual* | Tooth type | Average | SD | Average | SD | Ampl 44 |
| ***CI-E1 AdultoB*** | **C** | **-12.82** | **0.09** | **-2.81** | **0.16** | **866** |
| *CI-E1C* | dC | -12.41 | 0.06 | -2.34 | 0.05 | 1885 |
| *CI-E1C* | $M_1$ | -12.96 | 0.05 | -3.25 | 0.13 | 1015 |
| *CI-E1D* | $M_1$ | -13.40 | 0.04 | -3.49 | 0.06 | 2895 |
| *CI-E21* | dI | -12.38 | 0.04 | -6.40 | 0.05 | 4072 |
| *CI-E21* | $M_1$ | -11.33 | 0.06 | -3.18 | 0.09 | 2258 |
| | | | | | | |
| *CI-E21B* | dM | -11.51 | 0.08 | -3.53 | 0.08 | 2524 |
| *CI-E21C* | C | -12.31 | 0.17 | -2.40 | 0.15 | 1573 |
| ***CI-E21D*** | **$M_1$** | **-11.79** | **0.30** | **-2.38** | **0.36** | **327** |
| *CI-E21E* | $M_1$ | -12.84 | 0.08 | -2.84 | 0.09 | 1202 |
| ***CI-E21F*** | **$M_1$** | **-13.03** | **0.13** | **-2.97** | **0.18** | **797** |
| *CI-E28* | dM | -11.36 | 0.05 | -3.39 | 0.06 | 3557 |
| *CI-E29* | dC | -11.87 | 0.05 | -1.46 | 0.07 | 2812 |
| *CI-E29* | $M_1$ | -10.93 | 0.06 | -2.96 | 0.11 | 1457 |
| *CI-E29A* | dM | -12.00 | 0.07 | -5.35 | 0.08 | 1693 |
| *CI-E30* | dI | -12.00 | 0.10 | -3.53 | 0.10 | 1800 |
| *CI-E30* | $M_1$ | -13.73 | 0.16 | -4.65 | 0.13 | 1062 |
| *CI-E30A* | dM | -10.93 | 0.06 | -2.43 | 0.07 | 2565 |
| *CI-E31* | dM | -11.90 | 0.09 | -3.39 | 0.15 | 1916 |
| *CI-E35* | dI | -12.99 | 0.09 | -3.38 | 0.08 | 2195 |
| *CI-E35* | $M_1$ | -12.18 | 0.09 | -2.32 | 0.12 | 1200 |
| *CI-E35A* | dM | -12.63 | 0.12 | -4.68 | 0.16 | 992 |
| *CI-E36A* | dM | -12.77 | 0.11 | -6.23 | 0.14 | 1028 |
| *CI-E36A* | $M_1$ | -12.67 | 0.13 | -3.28 | 0.10 | 1244 |
| ***CI-E39*** | **$M_1$** | **-12.48** | **0.18** | **-3.98** | **0.14** | **735** |
| *CI-E39* | dM | -11.78 | 0.06 | -4.28 | 0.08 | 2426 |
| *CI-E40A* | dM | -11.33 | 0.03 | -2.36 | 0.07 | 2803 |
| *CI-E49* | dI | -13.10 | 0.08 | -3.50 | 0.12 | 2127 |
| *CI-E49* | dM | -13.02 | 0.07 | -2.42 | 0.12 | 1576 |
| *CI-E49A* | $M_1$ | -12.88 | 0.05 | -2.92 | 0.06 | 2141 |
| *CI-E51A* | dM | -12.75 | 0.06 | -3.21 | 0.05 | 2741 |
| *CI-E6* | dM | -12.73 | 0.08 | -4.98 | 0.09 | 1389 |
| *CI-E6* | dI | -12.69 | 0.06 | -4.60 | 0.08 | 2239 |
| *CI-E6* | $M_1$ | -11.57 | 0.05 | -2.93 | 0.09 | 2551 |
| ***CI-EE*** | **$M_1$** | **-11.72** | **0.10** | **-2.23** | **0.11** | **909** |
| *CI-EE* | dM | -12.84 | 0.09 | -1.12 | 0.10 | 2108 |
| ***CI-I22*** | **$M_1$** | **-11.82** | **0.09** | **-2.75** | **0.09** | **838** |
| *CI-I23* | $M_1$ | -12.71 | 0.11 | -3.65 | 0.12 | 1279 |
| ***CI-I25*** | **$M_1$** | **-12.57** | **0.09** | **-3.62** | **0.13** | **700** |
| ***CI-I26*** | **$M_1$** | **-12.90** | **0.22** | **-3.34** | **0.30** | **757** |
| ***CI-I27*** | **$M_1$** | **-12.53** | **0.09** | **-4.32** | **0.14** | **837** |
| ***CI-I28*** | **$M_1$** | **-12.30** | **0.21** | **-3.55** | **0.20** | **556** |
| ***CI-I29*** | **$M_1$** | **-12.65** | **0.15** | **-3.36** | **0.20** | **726** |
| *CI-I31* | $M_1$ | -12.39 | 0.10 | -2.21 | 0.09 | 1317 |
| ***CI-I40*** | **$M_1$** | **-13.46** | **0.20** | **-4.28** | **0.22** | **513** |
| ***CI-I41*** | **$M_1$** | **-12.24** | **0.04** | **-3.24** | **0.13** | **829** |
| *CI-I43* | $M_1$ | -13.09 | 0.10 | -2.08 | 0.07 | 1754 |
| *CIE34A* | dM | -12.32 | 0.05 | -1.68 | 0.10 | 1888 |
| *MII-E21* | dM | -10.59 | 0.10 | -2.67 | 0.14 | 1227 |

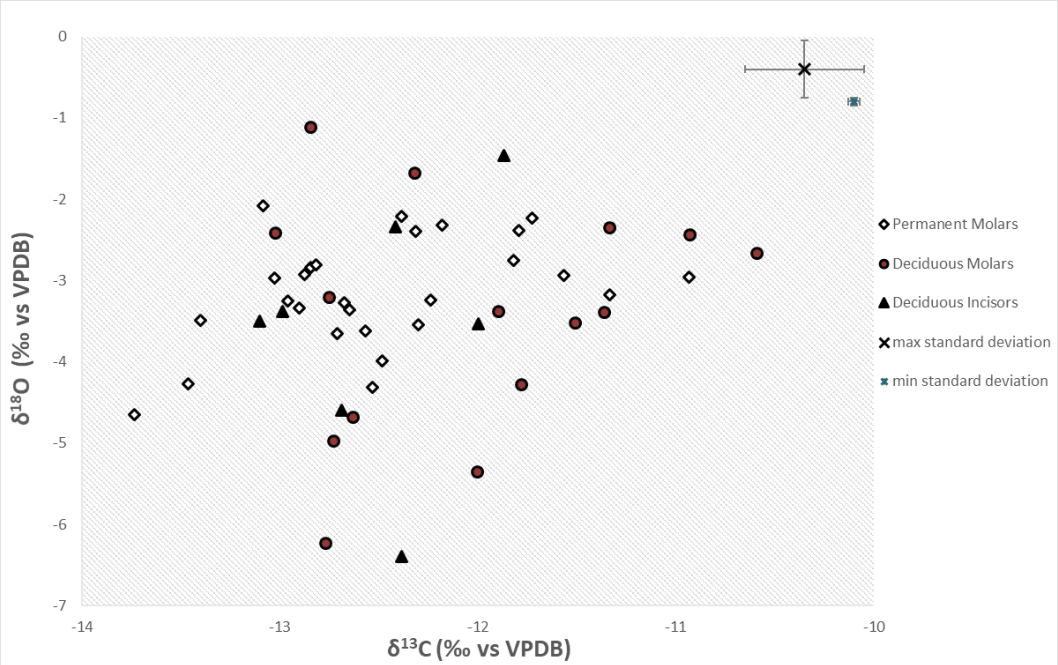



Figure 9 – Scatter plot of the $\delta^{18}O$ and $\delta^{13}C$ values of dental enamel from all 34 individuals sampled from the site of Cueva del Infierno, Cuba.

These three groups, dI, dM and M, will be used in data analysis and interpretations.

As shown in Figure 9, the values are scattered for all three groups with no particular distinction among them, regarding either $\delta^{18}O$ or $\delta^{13}C$. What is noticeable is the narrower range of the deciduous incisor $\delta^{13}C$ values (1.2‰) compared to the ranges of the other groups (2.4‰ for the dM group and 2.8‰ for the $M_1$ group) (Table 4). Furthermore, it is visible that only permanent teeth have $\delta^{13}C$ values lower than -13.1‰. It is also clear that although the $\delta^{18}O$ values for deciduous teeth scatter along the y-axis (with ranges around 5‰ for both dM and dI group), the permanent teeth have relatively higher $\delta^{18}O$ signatures (>-4.7 ‰) with a noticeably narrower range of ratios (2.6‰). Thus, it could be implied that in younger ages the $\delta^{13}C$ values that represent the diet of the individual are less variable than older children. However, the opposite occurs with the water intake that is depicted by $\delta^{18}O$ ratios. Finally, older children have a more restricted range of values from -4.7‰ up to -2.1‰, compared to younger children whose values fluctuate in a broader range.

Table 4- Minimum values, maximum values and ratio ranges for $\delta^{13}C$ and $\delta^{18}O$ isotopic signatures for the three different tooth types with their number of samples per element. Calculations based on the overall sample of individuals (n=34)

| | *dI* | | *dM* | | *$M_1$* | |
|---|---|---|---|---|---|---|
| | **$\delta^{13}C$‰** | **$\delta^{18}O$‰** | **$\delta^{13}C$‰** | **$\delta^{18}O$‰** | **$\delta^{13}C$‰** | **$\delta^{18}O$‰** |
| *Minimum value* | -13.1 | -6.4 | -13.0 | -6.2 | -13.7 | -4.7 |
| *Maximum value* | -11.9 | -1.5 | -10.6 | -1.1 | -10.9 | -2.1 |
| *Range* | 1.2 | 4.9 | 2.4 | 5.1 | 2.8 | 2.6 |
| *Count* | 7 | | 15 | | 25 | |

### 4.1.2 Subgroup data analysis for both carbon and oxygen

The same type of graph was also created for the nine individuals from which multiple samples were taken (two or three teeth) that represent specific time periods of life (data shown on Table 5). The scatter plot in Figure 10 represents the measured values of $\delta^{18}O$ (‰ VPDB) on the y-axis arranged in correlation with the $\delta^{13}C$ (‰ VPDB) values on the x-axis. Once again, the three different groups of teeth, as divided previously, are also distinguishable in this graph.

The same patterns are noticeable for the subgroup of individuals, since it represents a smaller scale of the first one, where data from nine out of 34 individuals is shown. Relatively lower $\delta^{18}O$ values are observed for the deciduous teeth compared to the permanent molars, while the ranges of the $\delta^{13}C$ ratios follow the above diagram in Figure 9, with more restricted values depicted for the time *in utero* (dI).

Once the values of carbon and oxygen were observed in relation to one another, more information needed to be extracted, mostly regarding the changes occurring among the teeth categories. The examination of the oxygen and carbon intake differences were analyzed and studied separately for all 34 individuals, as well as for the ones that were able to be sampled for more than one tooth (subgroup, *n*=9).

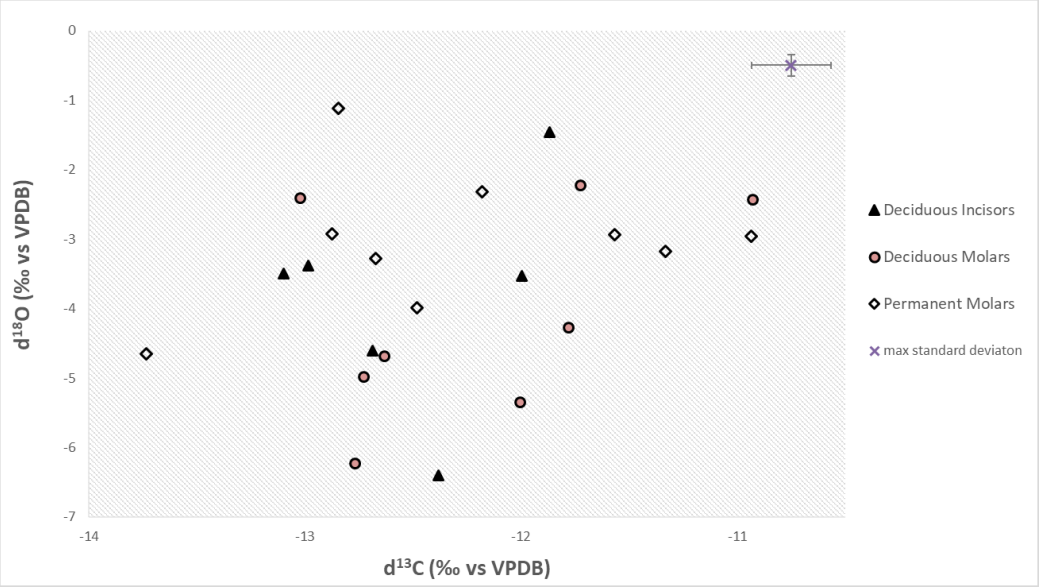



Figure 10 – Scatter plot of the $\delta^{18}O$ and $\delta^{13}C$ values of the selected multi-sampled individuals from Cueva del Infierno population (n=9).

Table 5 –Carbon and oxygen isotopic signatures of the subgroup of the nine individuals that were multi-sampled. The table shows the individual, the tooth type and the $\delta^{18}O$ and $\delta^{13}C$ values expressed in ‰. In **blue** the ratios with less signal intensity.

| | Individual | Tooth type | $\delta^{13}C$ ‰ | $\delta^{18}O$ ‰ |
|---|---|---|---|---|
| **1** | E-49 | dI | -13.1 | -3.5 |
| | E-49 | dM | -13.0 | -2.4 |
| | E-49 | $M_1$ | -12.9 | -2.9 |
| **2** | E-39 | dM | -11.8 | -4.3 |
| | E-39 | $M_1$ | **-12.5** | **-4.0** |
| **3** | E-36A | dM | -12.8 | -6.2 |
| | E-36A | $M_1$ | -12.7 | -3.3 |
| **4** | E-35 | dI | -13.0 | -3.4 |
| | E-35 | dM | -12.6 | -4.7 |
| | E-35 | $M_1$ | -12.2 | -2.3 |
| **5** | E-30 | dI | -12.0 | -3.5 |
| | E-30 | dM | -10.9 | -2.4 |
| | E-30 | $M_1$ | -13.7 | -4.7 |
| **6** | E-29 | dC | -11.9 | -1.5 |
| | E-29 | dM | -12.0 | -5.4 |
| | E-29 | $M_1$ | -10.9 | -3.0 |
| **7** | E-21 | dI | -12.4 | -6.4 |
| | E-21 | $M_1$ | -11.3 | -3.2 |
| **8** | E-6 | dI | -12.7 | -4.6 |
| | E-6 | dM | -12.7 | -5.0 |
| | E-6 | $M_1$ | -11.6 | -2.9 |
| **9** | EE | dM | **-11.7** | **-2.2** |
| | EE | $M_1$ | -12.8 | -1.1 |

## 4.2 Carbon isotope analysis

### 4.2.1 Overall sample observations

Studying carbon isotopic values separately can give the opportunity to find and examine differences that will give insights about the pre-natal period compared to the breastfeeding period (early infancy). The first step was to create a general box plot for all 34 individuals (Figure 11). The statistical analysis used for the creation of the box plot is provided in Table 6. The $\delta^{18}O$ values were plotted in different boxes for the different teeth groups. On this graph the median of the values under study is depicted with a line within the box and the mean with an "x". The upper end of each box represents the Q1 value (25% of the data are greater than this value) and the lower end the Q3 value (25% of the data are less than this value). The maximum and minimum values (not including outliers) are also stated by the determined lines (whiskers) outside the boxes. Values that fall outside the lower and upper limits calculated are considered outliers and they are shown as separate dots (outside of the whiskers).

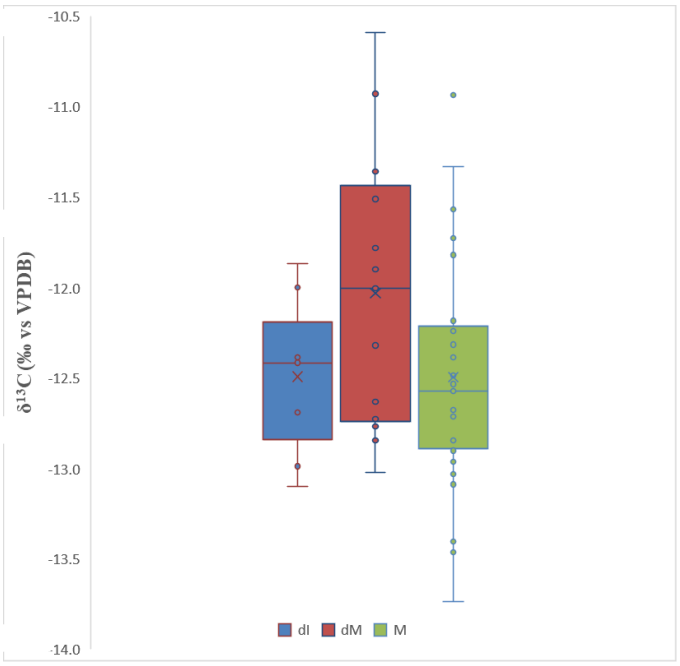



Figure 11 - Box plot of the $\delta^{13}C$ values of all 34 individuals sampled from Cueva del Infierno population. The three different boxes represent the three different tooth categories, dI, dM, $M_1$ with all the values displayed as labels.

Table 6 - Calculations needed for the creation of the box plot for the three different groups (n=34).

| | *dI* | *dM* | *M* |
|---|---|---|---|
| **Minimum** | -13.1 | -13.0 | -13.7 |
| **Q1** | -12.8 | -12.7 | -12.9 |
| **Median** | -12.4 | -12.0 | -12.6 |
| **Q3** | -12.2 | -11.4 | -12.2 |
| **Maximum** | -11.9 | -10.6 | -10.9 |
| | | | |
| **Mean** | -12.5 | -12.0 | -12.5 |
| **Range** | 1.2 | 2.4 | 2.8 |
| | | | |
| **IQR** | 0.6 | 1.3 | 0.7 |
| **IQR X 1,5** | 1.0 | 2.0 | 1.0 |
| **Lower limit** | -13.8 | -14.7 | -13.9 |
| **Upper limit** | -11.2 | -9.5 | -11.2 |

In the box plots it is noticeable that the range of the carbon isotopic values for the individuals *in utero* is narrower compared to the ranges of the dM group and is smallest for the $M_1$ group. This was expected as it follows the pattern that was observed previously in the scatter plots. What is interesting here is the movement of the range of the signatures from the dM group to the $M_1$ group. According to the box plot, both the mean and median values of these groups are becoming more negative through time (keeping in mind that the order of the boxes shown in the graph follows the chronological order in which the teeth under study develop). Therefore, the study of the overall group of 34 individuals shows that the carbon isotopic values become less negative after birth and then start to decrease while the first permanent molar is developing.

### 4.2.2 Subgroup observations

The $\delta^{13}C$ values were also studied separately for the nine multi-sampled individuals. Two graphs were created, one that represents the $\delta^{13}C$ values of the subgroup of nine individuals in a box plot with the data presented in the same three groups as previously (dI, dM, $M_1$) (Figure 12) and one comparing only the deciduous incisors and first permanent molars of the six individuals that had dI available for sampling (Figure 13). Additionally, a scatter plot was created with the individual carbon isotope ratios from each group plotted on the y-axis (Figure 14) with the aim to ease comparison among the ranges and the location and/or accumulation of the values through the axis.

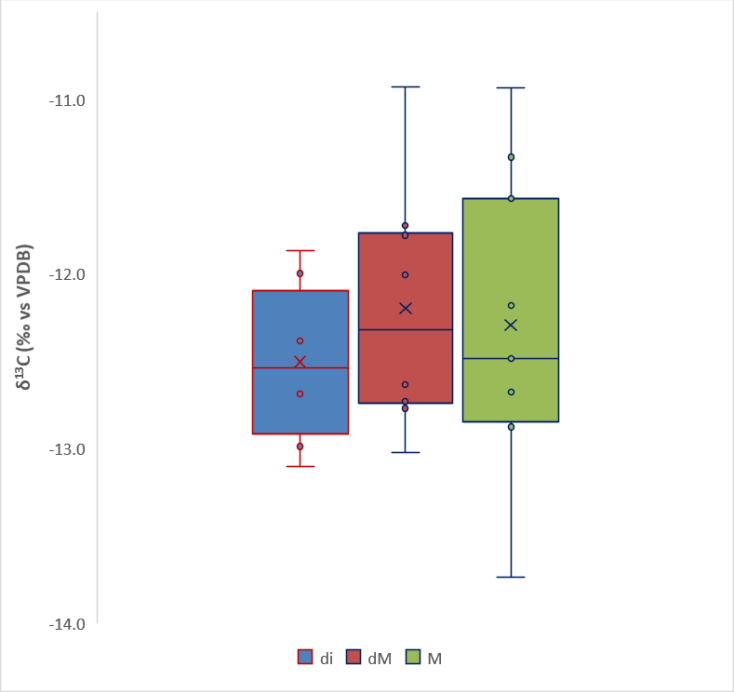



Figure 12 – Box plot of the d$^{13}$C values of the subgroup of nine.

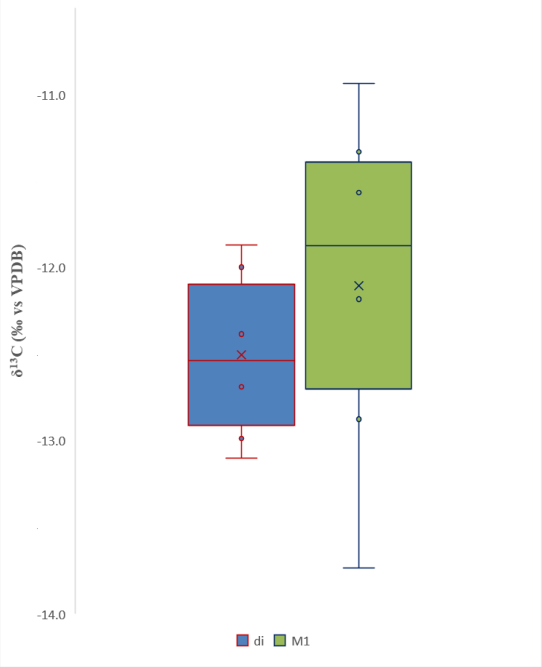



Figure 13 – Box plot of the dI $\delta^{13}C$ and $M_1$ $\delta^{13}C$ ratios (n=6).

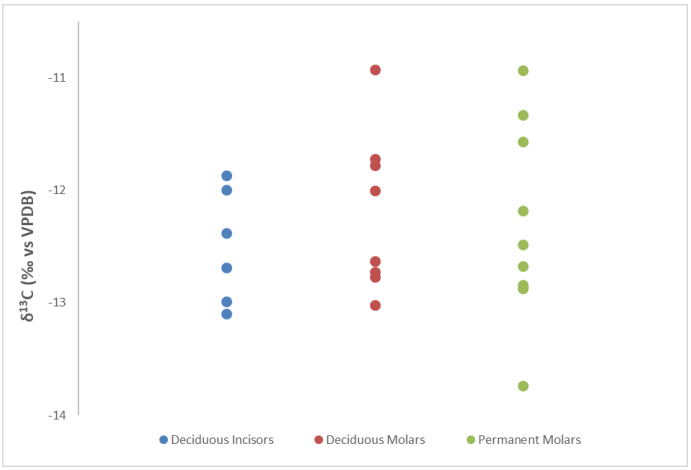



Figure 14 - Carbon isotopic values plotted on y-axis for the three different tooth categories (n=9).

After observation of the box plot it is apparent that the ranges of the $\delta^{13}C$ values are increasing together with age. Starting with the dI group the range is only 1.2‰, it broadens during dM development at 2.1‰ and gets even larger around two years of age at 2.8‰. It is also noticeable that excluding sample E-30 with a value of -13.7‰, the rest of the isotopic signatures are increasing for every tooth category with minimum $\delta^{13}C$ values at -13.0‰ for the dM group and -12.9‰ for the $M_1$ group.

### 4.2.3 Intra-individual observations

The two graphs, the one for the overall sample (Figure 11) and the one for the subgroup (Figure 12) show interesting differences of the isotopic change of values through time. For this reason, it was of interest to investigate the shifts that are happening to the isotopic values of each of the nine multi-sampled individuals separately. Consequently, nine intra-individual scatter plots were created including all three tooth types that might make visible possible patterns of alterations and fluctuations of the values (Figure 15).

The range of the isotopic values on the y axis remained the same, in order to enable relative observations. Five out of the six individuals that had a representative deciduous incisor in the sample show more negative dI $\delta^{13}C$ values compared to their respective $M_1$ and for only one the signatures decrease through time. The deciduous molar was sampled for only eight individuals and in a comparison between dM and $M_1$ groups, it can be observed that five out of eight have a lower dM $\delta^{13}C$ value compared to their $M_1$ $\delta^{13}C$ value. The $M_1$ ratios were decreased for three of these individuals. Furthermore, five triplets of teeth are available. In three out of these five individuals the carbon isotopic signatures increase to less negative values during the development of dM. For two of them (E-49, E-35) the values continue to increase through time but for one, E-30, the signature decreases again by 2.8‰, reaching a value even more negative than the one before birth during $M_1$ mineralization. The opposite occurs in two of the five, where the dM $\delta^{13}C$ values are more negative than the dI ratios and then raise again during the formation of the first permanent molar (E-29, E-6).

It is noticeable that with subsequent samples from the same individual the isotopic changes observed throughout a specific period of one's life are rather more complicated than a simple increase or decrease. The intra-individual scatter plots in Figure 15 show a variety of patterns, presenting mostly $\delta^{13}C$ values that increase in time, with other interesting fluctuations that will be further discussed in a later chapter.

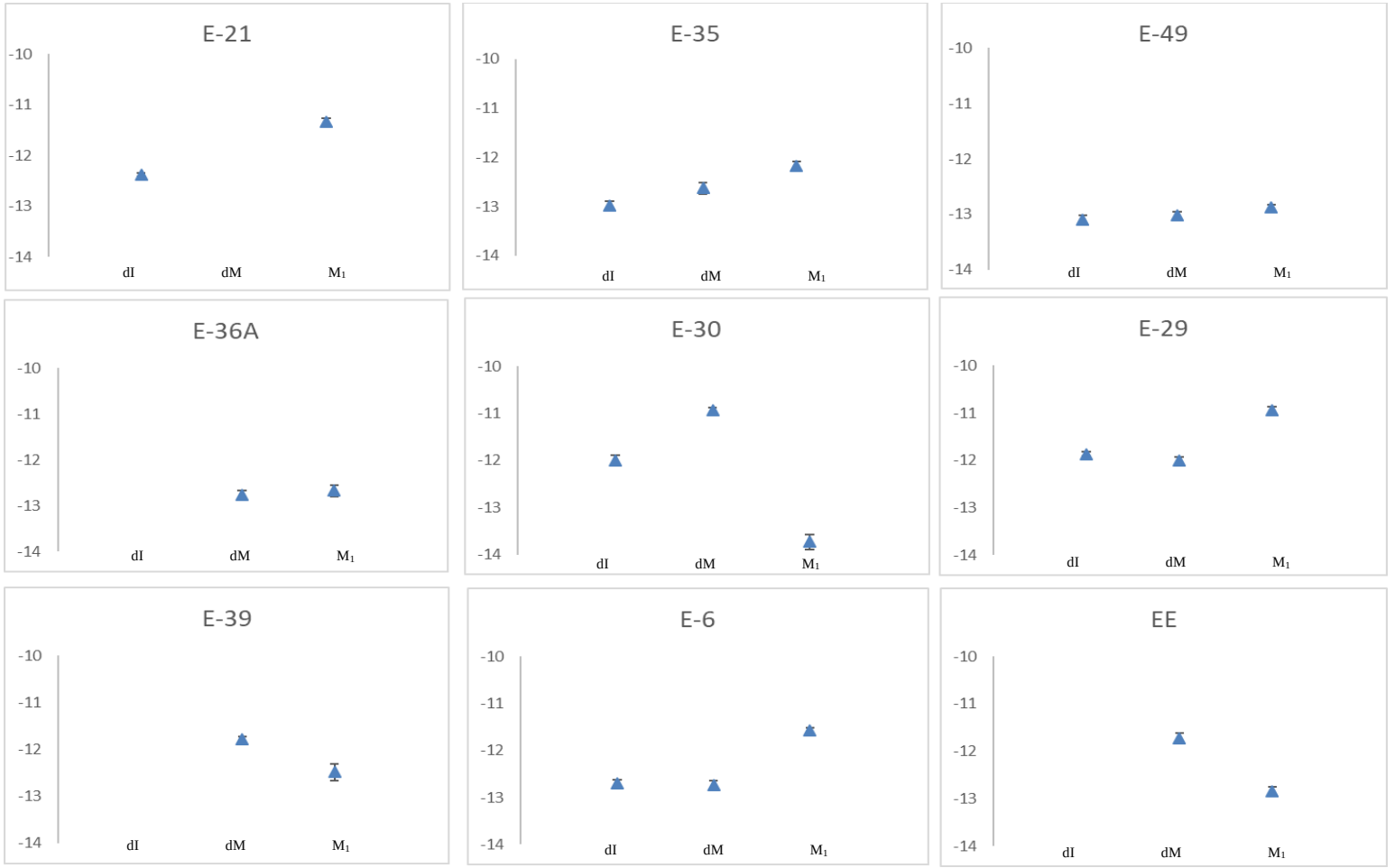



Figure 15 - Separate scatter plots for every individual of the subgroup that was multi-sampled, with each graph presenting the $\delta^{13}C$ values of each tooth that was sampled [δ13C (‰ vs VPDB)) on the y-axis, including standard deviation error bars for each value.

## 4.3 Oxygen isotope analysis

### 4.3.1 Overall sample observations

In the same way as carbon isotopic signatures discussed previously, oxygen values were also studied separately. The first step was to create a general box plot for all 34 individuals (Figure 16). The statistical analysis used for the creation of the box plot is provided in Table 7. The $\delta^{18}O$ values were plotted in different boxes for the different teeth groups.

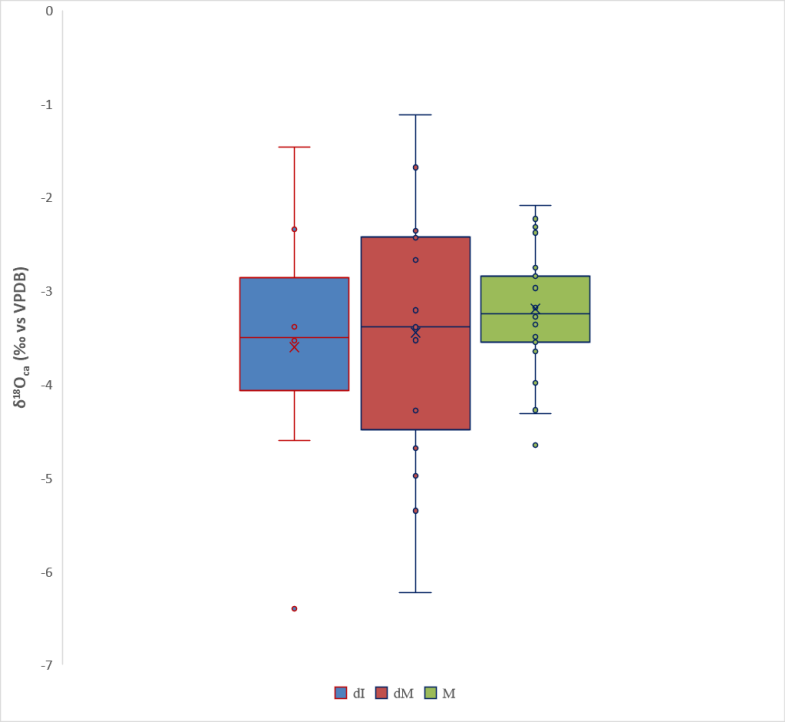



Figure 16 – Box plot of the $\delta^{18}O$ values of all 34 individuals sampled from Cueva del Infierno population. The three different boxes represent the three different tooth categories, dI, dM, $M_1$ with all the values displayed as labels.

Table 7 – Calculations needed for the creation of the box plot for the three different groups (n=34).

| | dI | dM | $M_1$ |
|---|---|---|---|
| **Minimum** | -6.4 | -6.2 | -4.7 |
| **Q1** | -4.1 | -4.5 | -3.5 |
| **Median** | -3.5 | -3.4 | -3.2 |
| **Q3** | -2.9 | -2.4 | -2.8 |
| **Maximum** | -1.5 | -1.1 | -2.1 |
| | | | |
| **Mean** | -3.6 | -3.4 | -3.2 |
| **Range** | 4.9 | 5.1 | 2.6 |
| | | | |
| **IQR** | 1.2 | 2.1 | 0.7 |
| **IQR x 1,5** | 1.8 | 3.1 | 1.1 |
| **Lower Limit** | -5.9 | -7.6 | -4.6 |
| **Upper Limit** | -1.1 | 0.7 | -1.8 |

In this case we can distinguish two outliers, one for the dI group (sample CI-E21 with a $\delta^{18}O$ value of -6.4‰) and one for the $M_1$ group (sample CI-E30 with a $\delta^{18}O$ value of -4.7‰). What else is noticeable in the box plot is that the range of values is largest for the dM group and smallest for the $M_1$ group. Furthermore, according to the box plot both the mean and median values of each group are gradually elevated through time (keeping in mind that the order of the boxes shown in the graph follows the chronological order in which the teeth under study develop).

Additionally, one extra graph was created with each oxygen isotopic value observable and plotted through the y-axis for each tooth category separately (n=34) (Figure 17). This allows better observation of the ratio ranges of the different groups, together with a representation of the relative location of the values through the y-axis. This graph shows once again how limited in extent the values of the permanent teeth are when compared to the deciduous teeth and the values considered as outliers are included. If the CI-E21 of the dI group is excluded, the differences between the ranges of the two groups are rather negligible.


Figure 17 – Oxygen isotopic values plotted on y-axis for the three different tooth categories (n=9).

### 4.3.2 Subgroup observations

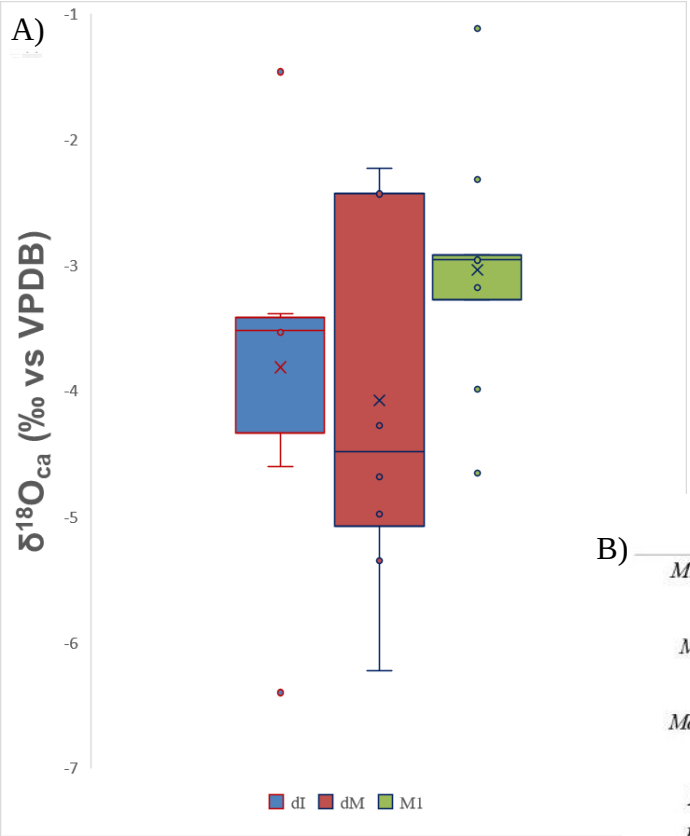
With the same strategy in mind, a box plot was also created for the $\delta^{18}O$ values of the nine multi-sampled individuals. The aim of this separate and targeted investigation is to examine the relation of ratios of three different tooth types taken from the same individual, which is the main focus of the present study. The box plot in Figure 18A is a concentrated representation of the $\delta^{18}O$ values from the different tooth categories of all nine individuals. Like previously, the mean value together with the median, Q1, Q2, minimum and maximum for each tooth type are noted and the statistics are provided in Figure 18B.

Furthermore, an additional scatter plot was created with the individual oxygen isotope values from each group plotted on the y-axis (Figure 18C). The aim of this graph is to ease comparison among the ranges and the location and/or accumulation of the values through the axis.

It is noticeable that without including what the box plot analysis considered an outlier, the $\delta^{18}O$ ratios of dI are lower, ranging from -3.4‰ to -4.6‰ compared to the ones of the permanent molars which range from -2.9‰ to -3.3‰. Even including the outliers, the majority of dI and dM values are located relatively lower than the signatures of the permanent first molars. Additionally, the dM values are spread in a broader range covering the ranges of dI and $M_1$ groups.

An additional effort was made to focus only on the six out of nine individuals (E-49, E-35, E-30, E-29, E-21, E-6) who had deciduous incisors available for sampling. The goal of this step is to study any particular differences between the dI group (which represents the prenatal period) and the $M_1$ group (which represents the postnatal period and thus the breastfeeding period of the child). This way, differences between before and after birth are more obvious and distinct. For this reason, a box plot was created for these six individuals, excluding the values from the dM group (Figure 19A). The statistics used for the formation of this boxplot are presented in Figure 19B.

A)




B)

| | *dI* | *dM* | *M1* |
|---|---|---|---|
| *Minimum* | -6.4 | -6.2 | -4.7 |
| *Q1* | -4.3 | -5.1 | -3.3 |
| *Median* | -3.5 | -4.5 | -3.0 |
| *Q3* | -3.4 | -2.4 | -2.9 |
| *Maximum* | -1.5 | -2.2 | -1.1 |
| | | | |
| *Mean* | -3.8 | -4.1 | -3.0 |
| *Range* | 4.9 | 4.0 | 3.5 |
| | | | |
| *IQR* | 0.9 | 2.6 | 0.4 |
| *IQR x 1,5* | 1.4 | 4.0 | 0.5 |
| *Lower Limit* | -5.7 | -9.0 | -3.8 |
| *Upper Limit* | -2.0 | 1.5 | -2.4 |

C)

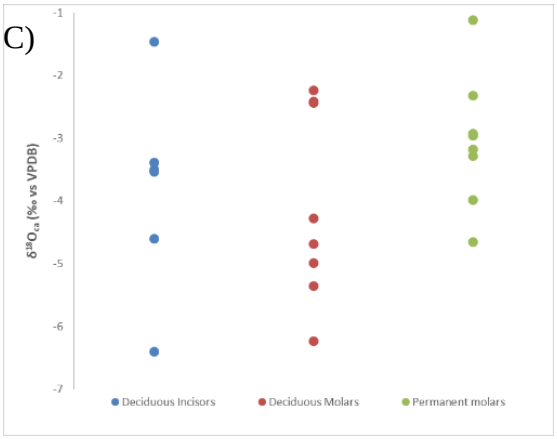
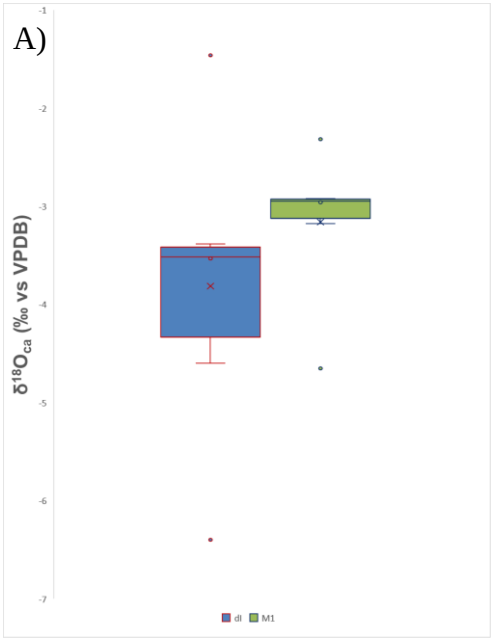



Figure 18 - A) box plot of the $\delta^{18}O$ ratios of the subgroup of the nine individuals that were multi-sampled. Each box represent a tooth category. B) the box plot analysis before the set up. C) the values plotted through the y-axis for better observation.

The graph in Figure 19 shows an even more distinct difference and elevation of the $M_1$ $\delta^{18}O$ values, compared to the dI group. There are four possible outliers, two for each tooth category. Excluding the potential outliers, the remaining four dI $\delta^{18}O$ signatures are relatively lower than the $M_1$ values. A variation like this would suggest higher $\delta^{18}O$ values during breastfeeding as it has been suggested in literature (Roberts et al. 1988, 625-627; Wright and Schwarcz 1998, 14).

A)




B)

| | *dI* | *$M_1$* |
|---|---|---|
| *Minimum* | -6.4 | -4.7 |
| *Q1* | -4.3 | -3.1 |
| *Median* | -3.5 | -2.9 |
| *Q3* | -3.4 | -2.9 |
| *Maximum* | -1.5 | -2.3 |
| *Mean* | -3.8 | -3.2 |
| *Range* | 4.9 | 2.3 |
| *IQR* | 0.9 | 0.2 |
| *IQR X 1,5* | 1.4 | 0.3 |
| *Lower Limit* | -5.7 | -3.4 |
| *Upper Limit* | -2.0 | -2.6 |

Figure 19 – A) box plot of the $\delta^{18}O$ ratios of the six out of nine individuals of the subgroup who had an available dI for sampling, together with the first permanent molar of each individual. Each box represents a tooth category. B) the box plot analysis before the set up.

#### *4.3.2.1 Statistical analysis of the difference observed between dI and $M_1$ group.*

The available $\delta^{18}O$ values of the two teeth categories, dI and $M_1$ and their means in a box plot showed an observable and noticeable difference. It is important to examine if this variance corresponds to a statistically significant difference or not. In this study the $\delta^{18}O$ signatures of the same six individuals are measured for the period prior and post birth. The null hypothesis ($H_0$) on this test is that there is no significant difference between the $\delta^{18}O$ signatures of the two groups and thus the results may correspond to random variation, while the $H_1$ hypothesis is that the difference of the data retrieved is significant enough to determine a pattern that is unlikely to be the result of random variation. The results of the paired t-test

performed t(5)=-0.9, p=0.4 (p>0.05) showed no significant difference between the two groups (data analysis in Table 8) and failed to reject $H_0$.

Table 8 – Paired-sample t-test statistical analysis for the $\delta^{18}O$ values of the two teeth categories, dI and $M_1$.

| | *dI* | *M* |
|---|---|---|
| *Mean* | -3,8 | -3,2 |
| *Standard deviation* | 2,6 | 0,6 |
| *Sample size* | 6 | 6 |
| *Pearson's correlation* | 0,06 | |
| *Alpha (a)* | 0.05 | |
| *Degrees of freedom* | 5 | |
| *P value one-sided* | 0.2 | |
| *P value double-sided* | 0.4 | |
| *t value* | -0.9 | |
| *t critical* | 2,5 | |

The pairing in dependent-sample t-tests gives statistical power as it reduces any possible variability between subjects. However, it can be susceptible to ordering effects. For this reason, a Wilcoxon Rank Sum test is used which is applicable for ordered-level data and it is the non-parametric equivalent of the paired t-test. This test combines the data of both groups and ranks them with the aim to detect clusters of values that may occur towards lower or higher scores, following the same individual through time. The data are supposed to be continuous and the test does not require a particular distribution of the dependent value (here the $\delta^{18}O$ ratios) since it is non-parametric. After application of the Wilcoxon Rank Sum test on the present sample, with the same null hypothesis as for the t-test previously, the results of the test w(6)=7, $w_{critical}$=0 showed that once again there was no significant difference between the groups and the null hypothesis failed to be rejected ($w > w_{critical}$, a=0.05).

### 4.3.3 Intra-individual observations

Even though a pattern of increased ratios was noticed in older age, the presence of the potential outliers does not allow for more specific and accurate evidence. This is why the $\delta^{18}O$ values are also plotted separately for the nine individuals in individual scatter plots including all three tooth types (Figure 20). This aimed to make any possible patterns of alterations and fluctuations of the values visible that are not able to be observed in the summary graphs. The analytical errors were also plotted for each value in order to give an indication of whether the observed differences may simply be the result of measurement uncertainty, as opposed to reflecting genuine differences.

The observations of the individual graphs can be focused in different comparisons. When comparing the relative position of the $\delta^{18}O$ ratios between dI and $M_1$ groups, two of the six individuals, which had a representative deciduous incisor in the sample had a higher dI $\delta^{18}O$ value compared to their $M_1$ $\delta^{18}O$ signature. On the other hand, four out of six had increased $M_1$ $\delta^{18}O$ ratios compared to the dI $\delta^{18}O$ from the same individual. In a comparison between dM and $M_1$ it is observed that six out of eight individuals which had a representative deciduous molar in the sample had a lower dM $\delta^{18}O$ value compared to their $M_1$ $\delta^{18}O$ value. The $M_1$ ratios were decreased only for two of these individuals. Unfortunately, in this sample there were only five individuals that were able to be sampled for all three tooth categories. In three out of five individuals (E-6, E-29, E-35) the isotopic ratio of oxygen decreases during the development of dM followed by an increase during the formation of the first permanent molar (Figure 20). The exact opposite pattern occurs in two of the six individuals (E-30, E-49), where the dM $\delta^{18}O$ values are higher than the dI ratios which then drop again during $M_1$ mineralization (Figure 20).

Ultimately, these intra-individual graphs show a variety of patterns. The most commonly observed being; a decrease of the $\delta^{18}O$ signatures during the development of the deciduous molars, lower ratios when comparing the time around birth and after and the increase of the $\delta^{18}O$ value after birth. Nevertheless, this model is not followed for all individuals and there were differential fluctuations, which do not allow for a general pattern to be established.

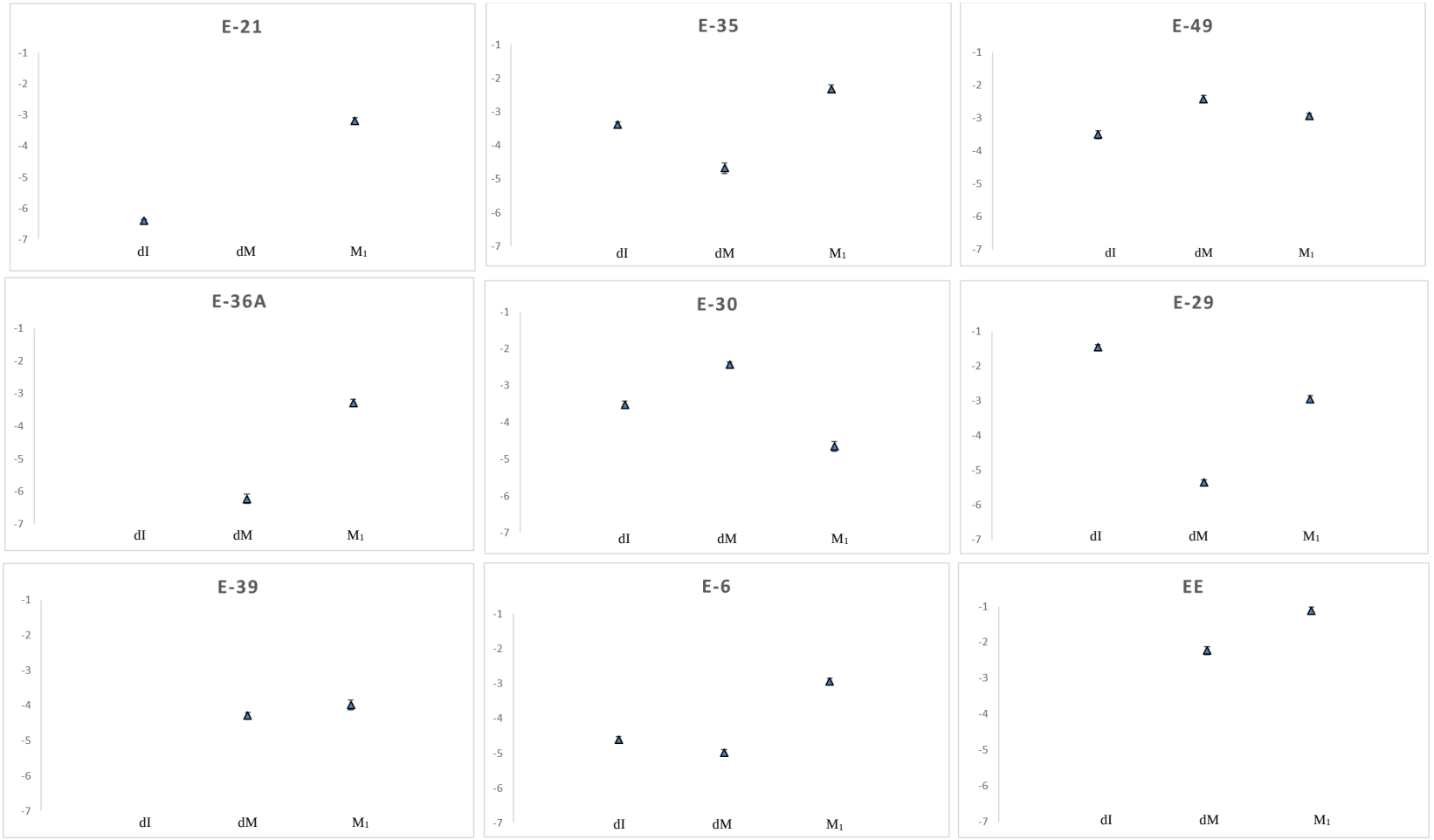



Figure 20 – Separate scatter plots for every individual of the subgroup that was multi-sampled, with each graph presenting the $\delta^{18}O$ values of each tooth that was sampled [$d^{18}O$ (‰ vs VPDB) on the y-axis including standard deviation error bars for each value.

In summary, after plotting the data, the variety and complexity of the intra individual isotopic patterns are evident for both carbon and oxygen isotopic values. Therefore, the results presented in this chapter appear informative and interesting, as they may reflect differences in breastfeeding and weaning practices within the population. Potential explanations of these findings will be further discussed in the next chapter.

# 5 DISCUSSION

In this chapter the results will be placed into context and further interpreted. The findings of the dental enamel carbon and oxygen isotope analysis will be examined separately, with extensive focus on the multi-sampled individuals which are expected to offer insights towards the main research questions of this study. The isotopic signatures of both chemical elements will be studied for trends and patterns, examining also the support or deviation from previous research on breastfeeding and weaning practices. The lack of other local information about oxygen isotopic signatures within the frame of BWPs should be noted here, together with the limitations that can arise from this fact. The methodological limitations and contextual implications of the study will be explored, with errors and potential biases considered prior to concluding arguments about the data. Lastly, suggestions (both contextual and methodological) will be proposed for future studies that may contribute to enrich and broaden our knowledge, not only on the BWPs of Cueva del Infierno, but also the lives of the archaic populations of the Caribbean.

## 5.1 Carbon isotope analysis

### 5.1.1 Placing the population in context

Carbon isotope values retrieved from dental enamel are representative of the carbon isotopic signatures of the food consumed and then distributed through the biological system of the organism through blood. When measured, these isotopic ratios indicate the contribution of each plant category ($C_3$, $C_4$, CAM) in the diet of the individual. This way an image can be created about the whole diet of the population under study (Hillson 2005, 146; Katzenberg 2008, 416-417). In this experiment, most of the individuals sampled were infants – with a few adult exceptions – and the $\delta^{13}C$ values of the deciduous teeth, which develop *in utero*, reflect the mothers' food intake, which passes to the fetus through blood.

Due to fractionation, $\delta^{13}C$ values in dental carbonate differ from the $\delta^{13}C$ values of food consumed by the individual. The average $\delta^{13}C$ values of plant tissues in the Caribbean have been estimated at approximately -25.5‰ for $C_3$ plants (ranging from -21.1‰ to -30.2‰) and around -9.5‰ for $C_4$ plants (with a range from -8.6‰ to -12.5‰) (Pestle 2010, 223). Regression analysis based on the

differential routing of the macronutrients in the blood plasma predicts an enrichment of the $\delta^{13}C$ dental enamel values of 10.1 ± 0.2‰ (Fernandes et al. 2012, 298). Therefore, including this fractionation the dental enamel $\delta^{13}C$ ratios are expected to range from -10.8‰ to -20.3‰ for the consumption of exclusively $C_3$ plants and from 1.7‰ to -2.6‰ for exclusively $C_4$ plant intake.

The calibrated values above can be used to examine the average diet of the people of Cueva del Infierno. The enamel $\delta^{13}C$ values in the present study range from a minimum signature of -13.1‰ to a maximum value of -10.6‰. Therefore, according to the isotopic data and the general $C_3$ versus $C_4$ values in the Caribbean, it can be confidently assumed that the population of Cueva de Infierno based its diet predominantly on $C_3$ plants.

Considering the size of the island and the different populations that might have occupied it at the same time, it is interesting to also examine whether populations at different locations had different dietary habits and thus explore similarities in their diet with that of Cueva de Infierno. Previous studies on stable isotope analysis of prehistoric Cuban populations can be valuable for this kind of comparison.

The study of Chinique de Armas and colleagues has focused on four populations (Canimar Abajo, Guayabo Blanco, Cueva del Perico I and Cueva Calero), neighboring Cueva del Infierno (Figure 21) with indications of $C_4$ plant cultivation present only for Canimar Abajo site (Chinique de Armas et al. 2016, 25; Smith et al. 2018, 102-105). Comparing the isotopic values of the Canimar Abajo, Guayabo Blanco, Cueva del Perico I and Cueva Calero, more similarities can be found among the last three populations that were assigned a $C_3$-plant based diet. Unfortunately, the study includes samples of bone collagen while the present study examines dental enamel isotopic variation. Therefore, the results of the two cannot be used for a direct comparison of their isotopic values.


Figure 21 - Relative location of Cueva del Infierno, Canimar Abajo, Guayabo Blanco, Cueva del Perico I, Cueva Calero and El Chorro de Maíta sites in Cuba. Figure edited by the author after Chinique de Armas et al. 2016

However, the study of Laffoon and colleagues that is currently in press (Laffoon et al. in press) is the latest study that used samples derived from enamel from 69 individuals, providing valuable isotopic results from El Chorro de Maíta in Cuba, that can be compared with data from Cueva del Infierno. Although, individuals from both sexes and all age ranges were sampled, in the case of teeth the authors focused mostly on premolars, avoiding this way the effect of breastfeeding in their results. The overall results of the study indicate a mixed diet primarily based on $C_3$, with small contributions of $C_4$ plants and/or seafood. The enamel $\delta^{13}C$ signatures of the local individuals in that study ranged from -9.7‰ to -15.5‰ (Laffoon et al. 2013, in press; Valcárcel Rojas et al. 2011). These results were plotted together with the ones from the present study in Figure 22, in order to allow comparisons and examine whether the isotopic values from Cueva del Infierno and El Chorro de Maíta fall within the same range. The analytical errors of each value from the present study were included in the scatter plot, in order to indicate possible uncertainties in the measurements.

As shown in the graph all the individual $\delta^{13}C$ signatures from the Cueva del Infierno population fall within the range of the carbon isotopic values from the prehistoric cemetery of El Chorro de Maìta. Due to lack of outliers in this regard, it can be assumed that the population of Cueva de Infierno had a similar diet with this precolonial Cuban population, based mainly on $C_3$ plants.

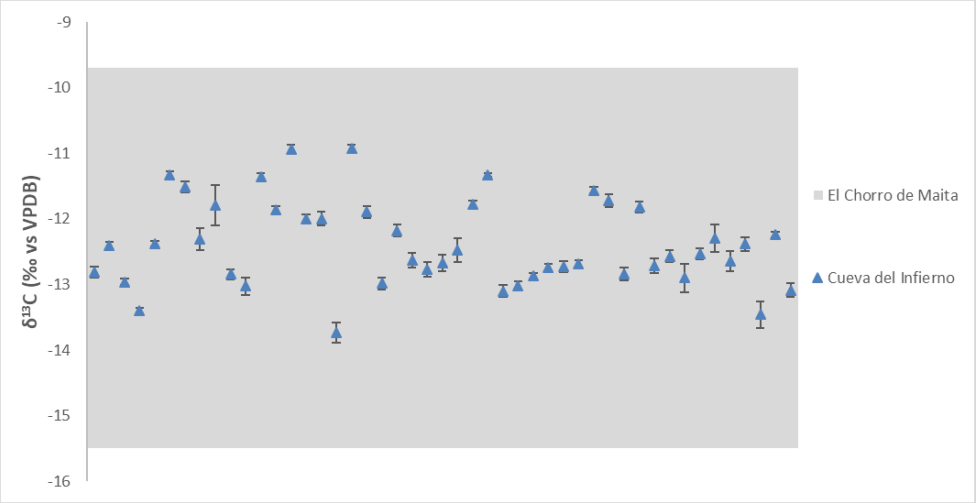



Figure 22 - Carbon isotopic signatures from Cueva del Infierno plotted related to the minimum and maximum $\delta^{13}C$ isotopic values from El Chorro de Maíta (grey area)(Laffoon et al. in press).

A $C_3$ plant-based diet appears to be the case for most of the precolonial Cuban populations that have been studied until present using isotope analysis with the only exceptions being Canimar Abajo and possibly El Chorro de Maíta. The comparison of the $\delta^{13}C$ signatures from Cueva del Infierno with other Cuban data (Chinique de Armas et al. 2016; Laffoon et al. in press) as well as with isotopic values from a broader region of the Caribbean shows that the consumption of the population under study focused primarily on $C_3$ plants.

### 5.1.2 Intra-individual trends in $\delta^{13}C$ signatures

Comparing the results among the different teeth categories from all 34 individuals, the general trend that can be observed is an increase of the isotopic signatures after birth (dM formation) followed by a decrease of values during the period of $M_1$ mineralization (Figure 11).

When focusing on the subgroup of the nine multi-sampled individuals, both slightly increased and decreased values are observed while comparing between dI and $M_1$ ratios (Figure 13). When deciduous incisors are compared with the first permanent molars, it is noticeable that the maximum values of the groups increase by 1‰ (from -11.9‰ for the dI group to -10.9‰ for the $M_1$) and the minimum values decrease slightly from -13.1‰ for the dI to -13.7‰ for the $M_1$ group. Additionally, the ranges of the $\delta^{13}C$ signatures are gradually broadened from 1.2‰ for the deciduous incisors to 2.8‰ for the permanent first molars, indicating an alteration occurred during the time of $M_1$ mineralization. The general trend in this

case does not follow the one observed in the overall sample, with the only indication of such a pattern of variation suggested by the mean values of each box plot of the subgroup.

It is apparent that the trends suggested by the box plots of the overall sample are slightly different than those of the subgroup. However, closer study of the subsequent samples from the same individual can provide more detailed information. When focusing on the separate graph of each of these individuals, a general increase of the $\delta^{13}C$ ratios through the years is observed for six individuals (E-36A, E-29, E-35, E-21, E-6 and E-49) and a decline is noticed in three of them (EE, E-39 and E-30). When dI is compared with $M_1$, the amount of increase ranges from 0.2‰ to 1.1‰. On the other hand, the decrease ranges from 0.7‰ to 1.7‰. Therefore, when examining the isotopic changes within the same individual through the years the most frequent pattern is a gradual increase that is usually bigger during $M_1$ mineralization (Figure 15).

Studies have shown that during the introduction of the first solid or liquid food to the infant (supplementation – start of weaning process), $\delta^{13}C$ values can show gradual enrichment with the increase of age (Wright and Schwarcz 1998, 12). Although isotopic data on weaning are limited for Cuba, there is one study which has estimated the start of the weaning process at around two years of age in Archaic Cuban populations based on carbon and nitrogen isotopic signatures. In the same study it is also noted that ethnographic data support the introduction of supplements in even earlier ages (Chinique de Armas and Pestle 2018, 506). Most studies outside the Caribbean show that supplementary food is more commonly introduced around the sixth month of the child's life (Izurieta and Larson-Brown 1995; Sellen 2001), with examples spanning from the third month (Rivera and Ruel 1997) to the first year of age (Dettwyler and Fishman 1992; Wright and Schwarcz 1998). The enamel formation in the first permanent molars starts after birth and completes after the second year of age, which means that the mineralization period most probably includes the initial supplementation of the child (AlQahtani et al. 2010, 485; Wright and Schwarcz 1998, 12). Considering these facts, the trend of the increased $\delta^{13}C$ values observed for some of the first permanent molars in this study can be interpreted as the indication of a shift that had begun towards other nutrient sources (instead of exclusively breastfeeding), reflecting an initial stage of supplementation.

Moreover, independent from the potential of early introduction of weaning food, there is also the possibility that the variation of the $\delta^{13}C$ values between the two groups reflects a difference between maternal diet (which would be represented by the dI group) and breast milk (which would be represented by the $M_1$ group). Studies on humans were not found, but comparable isotopic data from other mammals show alterations between the $\delta^{13}C$ ratios of lactation milk and the animal's diet, with values either decreasing (Boutton et al. 1988) or increasing (Mitchell et al. 1993). This difference can be explained by the higher intensity of body store usage for milk production during the lactation period (Wright and Schwarcz 1998, 11). Since no specific answer can be given as a pattern of alteration in this regard, the above phenomenon can be a possible explanation, considering that the range of the $\delta^{13}C$ values is broadening towards both sides.

Finally, regarding the values that seem to decrease in the sample with the increase of age, there is one more point that should be considered. The decreased part of the $\delta^{13}C$ values in the $M_1$ group, since it is considerably slight, can be explained by the fractionation of carbon in breast milk synthesis. More precisely, the majority of carbon in breast milk comes from its lipids, which constitute 33% of its dry part. Generally, breast milk lipids are expected to be isotopically lighter than other nutrients (Tiezen and Farge 1993, 123). According to these facts, it can be expected that infants that were exclusively breastfed will have slightly lighter $\delta^{13}C$ signatures that one that receives supplementary food (Wright and Schwarcz 1998, 11-12). If this is true, then in the cases where $\delta^{13}C$ enamel signatures are depleted during breastfeeding, they will be increased during supplementation.

## 5.2 Oxygen isotope analysis

### 5.2.1 Placing the population in context

The first step of the interpretation of the oxygen isotopic values for this experiment is to convert the dI $\delta^{18}O$ values (that represent the maternal water intake) to the corresponding drinking water ($\delta^{18}O_{dw}$) signatures and compare them with other $\delta^{18}O_{dw}$ isotopic data from Cuba.

Due to fractionation, $\delta^{18}O_c$ values in enamel carbonate vary from the $\delta^{18}O_{dw}$ values of the local water ingested. Generally, for the determination of the oxygen component in enamel, values derived from phosphate oxygen are considered more robust. For this reason, the enamel carbonate values ($\delta^{18}O_c$) can be converted to enamel phosphate ratios ($\delta^{18}O_p$) using a conversion equation (3) (Chenery et al. 2012, 313). The error generated by this conversion is not greater than the error indicated in phosphate analysis itself, thus it is not believed to introduce additional uncertainty to the converted values (Chenery et al. 2012, 316). In order to be able to calculate the drinking water values ($\delta^{18}O_{dw}$) of the individuals without converting them first to $\delta^{18}O_p$, Chenery and colleagues modified the equation of Daux and colleagues resulting to one that can be used directly for enamel carbonate ratios (4) (Chenery et al. 2012, 316; Daux et al. 2008, 102) .

$$\delta^{18}O_p = 1.0322(\pm 0.008) \times \delta^{18}O_c - 9.6849(\pm 0.187) \quad (3)$$

$$\text{Drinking water} = 1.590 \times \delta^{18}O_{VSMOW} - 48.634 \quad (4)$$

The above can be used to examine whether the $\delta^{18}O$ of the people of Cueva del Infierno correspond to the local water isotopic signatures. These conversions were applied to the $\delta^{18}O$ signatures of the dI group, since these teeth mineralize *in utero* and reflect the water drunk by the mother, avoiding breastfeeding and weaning practices input in the values. The enamel $\delta^{18}O$ values of the deciduous incisors in this study range from a minimum ratio of -6.4‰ to a maximum value of -1.5‰. The equations above use VSMOW as reference material for their isotopic values. This study uses as a reference material the VPDB (section 3.5, p.45), so the

first step is to convert the $\delta^{18}O_{VRDB}$ to $\delta^{18}O_{VSMOW}$. This is possible using the equations (5) and (6) below (Coplen 1988, 295).

$$\delta^{18}O_{VSMOW} = 1.0309 \times \delta^{18}O_{VPDB} + 30.91 \quad (5)$$

$$\delta^{18}O_{VPDB} = 0.97001 \times \delta^{18}O_{VSMOW} - 29.99 \quad (6)$$

The minimum $\delta^{18}O_{VSMOW}$ of this study was converted using equation (5) at 24.3‰ and the maximum at 29.4‰. Using equation (4) on these $\delta^{18}O_{VSMOW}$ values, the range of the drinking water signatures ($\delta^{18}O_{dw}$) for Cueva del Infierno individuals is calculated at -1.9‰ to -10‰ (Table 9A).

Table 9 – Conversions of the $\delta^{18}O_{VPDB}$ signatures of the two populations (Cueva del Infierno and El Chorro de Maíta) to $\delta^{18}O_{VSMOW}$ and drinking water values ($\delta^{18}O_{DW}$) using the equations (5) and (4).

A)

| | Individual | Tooth type | $\delta^{18}O_{VRDB}$ ‰ | $\delta^{18}O_{VSMOW}$ ‰ | $\delta^{18}O_{dw}$ ‰ |
|---|---|---|---|---|---|
| Cueva del Infierno | E-49 | dI | -3.5 | 27.3 | -5.2 |
| | E-35 | dI | -3.4 | 27.4 | -5.0 |
| | E-30 | dI | -3.5 | 27.3 | -5.3 |
| | E-21 | dI | -6.4 | 24.3 | -10.0 |
| | E-6 | dI | -4.6 | 26.2 | -7.0 |
| | E-29 | dC | -1.5 | 29.4 | -1.9 |
| El Chorro de Maíta | Local | max | -2.0 | 28.8 | -2.8 |
| | Local | min | -5.4 | 25.3 | -8.3 |

B)

| | Median ‰ | Mean ‰ | ±SD ‰ |
|---|---|---|---|
| Havana Province | -2.15 | -2.93 | 2 |

Furthermore, Laffoon and colleagues in their study (currently in press) have comparable enamel isotopic values available of 69 individuals from the precolonial El Chorro de Maíta site in Cuba (Laffoon et al. in press). Individuals from both sexes were sampled for this study and in the case of teeth the authors focused mostly on premolar samples, avoiding this way the effect of breastfeeding input in their results. For this comparison in this study, the maximum and minimum values of the 53 individuals regarded as locals were used. The enamel $\delta^{18}O_{VPDB}$ signatures of the local individuals ranged from -2‰ to -5.4‰. (Laffoon et al. in press). The conversions of these values to $\delta^{18}O_{dw}$ signatures are also included in Table 9A.

Moreover, data from the Global Network of Isotopes in Precipitation (GNIP) suggest that the range of the $\delta^{18}O_{dw}$ signatures of the island of Cuba in the Havana Province area is estimated using the maximum and minimum values at approximately 1‰ to -7‰ (VSMOW) (mean ± 2SD) (IAEA/WMO 2019) (Table 9B).

The precipitation and converted data from both Cuban sites are plotted in Figure 23, to allow comparisons among them and examine whether the isotopic drinking water values of Cueva del Infierno fall within the same range of El Chorro de Maíta and Havana Province area.

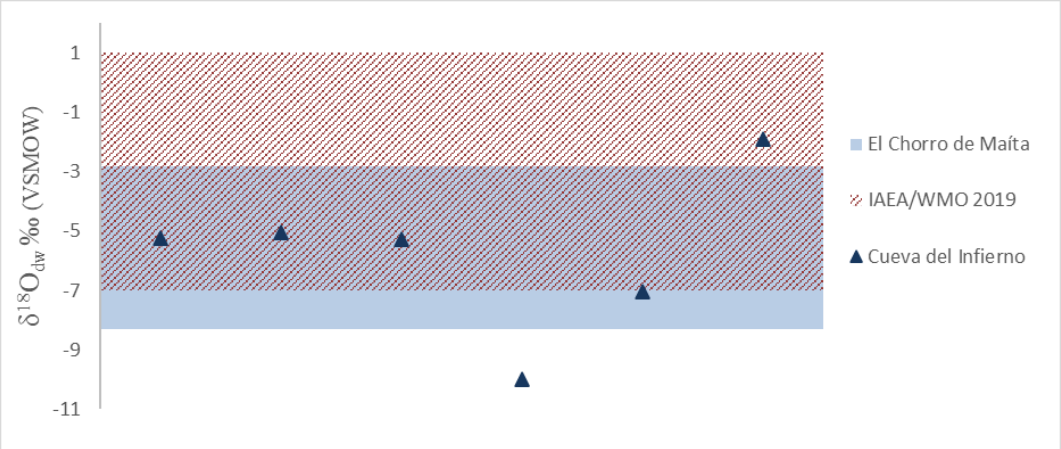



Figure 23 - Drinking water isotopic signatures from Cueva del Infierno plotted related to the minimum and maximum $\delta^{18}O_{dw}$ values from El Chorro de Maíta (grey area)(Laffoon et al. in press) and precipitation data of Havana Province area of Cuba (red striped area) (IAEA/WMO 2019).

The precipitation data derives from an area very close to the site under study and the data from El Chorro de Maíta regard local individuals. Although the greatest extent of the El Chorro de Maíta values overlap with the precipitation dataset, the later covers a broader range. Therefore, the combination of the both ranges can be assumed to represent local Cuban people. Three individuals from Cueva del Infierno fall within both ranges. Furthermore, one individual overlaps with the Havana Province range, one falls within the El Chorro de Maíta range of $\delta^{18}O_{dw}$ and one falls within the lower boarder of the IAEA/WMO 2019 data. Hence, it can be inferred that the oxygen isotopic values of drinking water for most of the individuals fall within the range of Cuban values. The only exception is individual E-21. The mineralization of the crown of the deciduous incisor occurs before birth, so the isotopic signature reflects the mother's diet. Therefore, an assumption can be

made that the mother of this individual may have migrated to Cueva del Infierno during her pregnancy. However, previous studies have shown that the $\delta^{18}O$ values in the Caribbean do not exhibit a great variation in general among the different islands (Laffoon et al. 2013, 7) so a variance of 1.7‰ can be attributed also to other practical factors. For this reason, more data should be recovered and more specifically strontium isotopic signatures for E-21, in order to make the argument of possible migration during pregnancy stronger.

### 5.2.2 Intra-individual trends in $\delta^{18}O$ signatures

In contrast with the $\delta^{13}C$ signatures, the $\delta^{18}O$ values presented in this study are highly variable, especially after closer examination of the intra-individual observations. It has been shown in previous studies that the $\delta^{18}O$ value of breast milk is higher than the $\delta^{18}O$ signature of the water drunk by the mother. Thus, oxygen isotopic signatures in the tissues of the infant are enriched during the process of breastfeeding. More explicitly, during breast milk intake, $\delta^{18}O$ ratios show a difference of 2-3‰. On the contrary, a relative decrease of the $\delta^{18}O$ ratios is expected during weaning, with a more obvious and distinct decline after the cessation of breastfeeding (Roberts et al. 1988, 625-627; Wright and Schwarcz 1998, 3). This has to do with the fact that this transition happens gradually, and breast milk meals are slowly replaced with normal food rather than a sudden exclusion. This translates to an alteration of the $\delta^{18}O$ signatures that is most commonly from 0.3‰ to 2‰ when weaning happens gradually, while more extensive differences are predicted when breastfeeding cessation is rapid (Roberts et al. 1988, 627).

On the other hand, there are studies that contradict or partially contradict the expected enrichment of the $\delta^{18}O$ values during breastfeeding. For instance, White et al. (2004, 244) found further enrichment occurring later in the child's life in their study of second permanent molars, meaning an observation of higher $\delta^{18}O$ values post-weaning. Based on these results, they argued that there is a potential that all teeth prior to weaning processes reflect the $\delta^{18}O$ values of the mother (White et al. 2004, 244). Wright and Schwarcz (1998, 13-14) found a combination of both increase and decline among the individuals in their study, similar to the patterns of variation presented here.

When the general graph of all 34 individuals analysed in this study is observed, there is no general trend except for the fact that the $\delta^{18}O$ values of the permanent teeth are more restricted in a narrower range in comparison with the deciduous ones (Figure 16, p.58). This possibly indicates a more constrained source of water around this time period of life which could indicate water intake through the mother's breast during breastfeeding. Other than that, there is no universal increase in the values of the permanent first molars, as would have been expected according to the published literature.

When the focus is turned towards the subgroup of individuals that were multi-sampled, the picture becomes complex. According to previous studies, it would be expected that the intra-individual graphs will show an increase from birth (deciduous teeth) towards two years of age (first permanent molars), while the child is assumed to have breastfed. In the results of this experiment, when comparing the values of deciduous molars with the permanent first molars, only six of the nine individuals show an increase of $\delta^{18}O$ during the first two years of life. Except for E-39 (0.3‰ increase) and EE (1.1‰ increase), the rest (E-36A, E-29, E-35, E-6) have a variance around 2-3‰ as would have been expected due to breastfeeding. One individual, E-21, lacks the dM for comparison, but shows an increase of 3.2‰ when the its dI is compared with its $M_1$. A general trend of increase in the $\delta^{18}O$ signatures is suggested when the group of dM is removed and only the deciduous incisors are compared with the first permanent molars (Figure 19, p.62). However, after statistical analysis, the increase in values was found to be not significant, due to the general inconsistency of the inter-individual patterns.

On the other side of the spectrum, two individuals, E-30 and E-49, show a $\delta^{18}O$ decline during the mineralization of $M_1$. This variation indicates that not all $M_1$ values necessarily reflect breastfeeding practices or that breast milk is not always enriched in relation to placental blood. There are different factors that may be responsible for the changes observed. Social or individual factors concerning the mother, or the infant can be included, and will be further examined in the following paragraphs.

As previously mentioned, weaning age is not fixed for all populations and/or individuals. The inconsistent patterns of the $\delta^{18}O$ values for the multi-sampled

individuals can possibly be explained by a variance in the timing of the shift from breast milk to weaning food. Early supplementation can play a role in the decreased values when comparing deciduous to permanent teeth ($M_1$s). Differences in age of supplementation among skeletal remains has also been shown in other studies, with the shift possibly occurring in different stages of life (Wright and Schwarcz 1998, 14). The observed lower $\delta^{18}O$ values could suggest earlier introduction to weaning foods for these children, occurring before the completion of $M_1$ mineralization. The mineralization of $M_1$ crowns last more than one and a half years. This way there is a possibility that different oxygen isotopic values from the same crown may reflect different feeding periods (breastfeeding and/or supplementation). The decrease observed in the two individuals is also not consistent with a 2.2‰ decline for E-30 but only a 0.5‰ decline for E-49. This fact can be attributed to a gradual meal replacement, with E-30 weaning potentially starting earlier than E-49. The $M_1$ $\delta^{18}O$ value of individual E-49 is lower than its dM, which would suggest that the child was breastfeeding in the first months of its life and then water was introduced, translating to a small $M_1$ $\delta^{18}O$ decline but still a higher value than the time *in utero* (dI). The same is not true for infant E-30, whose $\delta^{18}O$ value as a child around two years old is even lower than the one while *in utero*, although an increase took place in the first months of its life that could indicate breastfeeding. Moreover, the $\delta^{18}O$ value of $M_1$ is still within the general range of the population and the difference is not great, so the possibility of spatial mobility of the mother prior to birth is excluded.

Indications such as those above would suggest that the enrichment due to breastfeeding is obvious in the deciduous molars. These mineralize the first months after birth, while the infant most likely exclusively receives milk from the mother. Although two individuals, E-30 and E-49, show such results, with an increase of 1‰ during the mineralization of dM, there is again variation across the sample. Three individuals show differential decreases in values for the same period (3.9‰, 1.3‰, and 0.3‰ decline for E-29, E-35 and E-6, respectively). Four individuals lack one of the deciduous teeth, which limits further interpretations.

An alternative suggestion for the variations observed could be that cessation of breastfeeding came earlier or even more suddenly for some individuals due to a greater possibility of premature death. Although the effects of all diseases have not

been studied yet, the variation of the $\delta^{18}O$ signatures due to metabolic diseases has been examined (White et al. 2004). This inquiry showed non-significant differences in the isotopic values between individuals who suffered and people that did not (White et al. 2004, 243). However, studies have shown that maternal investment can differ for several reasons, including pathological indicators (White et al. 2004, 244). Signs of a diseased child can be an important factor for a mother to adopt different feeding practices, either with less attention to the process of breastfeeding or an earlier introduction to different nutrients. Considering most of the individuals under study here are children who died before their third year of life, this factor can be a possible explanation for the variation in the $\delta^{18}O$ ratios, reflecting different breastfeeding practices among the mothers. This fact relates directly with the osteological paradox, a limitation that will be discussed later in this chapter.

The possibility of the total exclusion of an infant from breastfeeding should also be considered. That could be attributed to a total lack of maternal investment, a non-lactating mother, the child refusing to breastfeed or sickness of the mother among many other possible reasons. Furthermore, there have been indications, also in the Caribbean, of differences in the age of weaning according to sex, with girls being weaned half a year or even a year earlier than boys (Fildes 1986, 45-66; Quinlan et al. 2005, 476). Such an argument would explain some of the variation observed in the present study, but the lack of information about the sex of the individuals does not allow further interpretations in this regard.

Seasonality can also play an important role in the variation of oxygen isotopic signatures, possibly overwriting breastfeeding signals. The $\delta^{18}O$ ratios of the environmental water can vary by location and by season, resulting in significant alterations of the $\delta^{18}O$ values in the individuals under study (Bryant et al. 1996; Gat 1996, 245). Since humans, unlike other mammals, do not mate seasonally, there is a possibility that some of the small fluctuations in the $\delta^{18}O$ values passed to the infants are due to differences in food availability and environmental water variability through the year among the mothers. The $\delta^{18}O$ signatures in the blood of a pregnant woman or a lactating mother can be susceptible to changes due to seasonality. Additionally, season of birth and death can also have an effect in the oxygen isotopic signatures of infants during their growth and the mineralization of their tissues (Britton et al. 2015, 235-236).

## 5.3 Carbon and oxygen signatures combined

At this point it is interesting to examine the relation among the individuals that show signs of early supplementation for each element. For oxygen these are samples E-49 and E-30 (Figure 20, p.65), and the start of the weaning process is indicated by the decline of the $\delta^{18}O$ values. On the other hand, supplementation is indicated by an increase in the $\delta^{13}C$ ratios. Between these two individuals, the isotopic signatures of both chemical elements agree towards alternative nutrient intake for sample E-49, where $\delta^{13}C$ signatures are gradually elevated through time (Figure 15, p.57) while $\delta^{18}O$ shows a depletion during the mineralization of $M_1$ (Figure 20, p.65), making the argument of early supplementation stronger. According to the anthropological report (Ruiz and Beatón, 2000) the estimated age of death for this infant ranges from six months to one and a half years. If the argument of early supplementation is true, then weaning is indicated from both carbon and oxygen isotopic signatures somewhere after the fifth month of life.

Individual E-30 shows the same pattern of isotopic change for both elements with a decrease for both $\delta^{13}C$ and $\delta^{18}O$ signatures in older ages. Generally, oxygen isotope values indicate water intake and carbon isotope demonstrates food consumption. Comparing the deciduous molars with the permanent first molars the $\delta^{13}C$ signature is 2.8‰ lower (Figure 15) and the $\delta^{18}O$ value declines by 2.2‰ (Figure 20). Since both differences appear to be considerable and, the age of death assigned for this individual is approximately six months old, one possible interpretation can be that while the infant was breastfeeding (indicated by the decreased values of carbon) it might have received a liquid form of supplementary food (indicated by the variation in oxygen values). The examination of the isotopic signatures of individual E-30 may indicate that the identification of (liquid) supplementation is possible when the isotopes of different chemical elements are studied. However, there are other possible explanations for mismatching value alterations. Although the expected procedure is a healthy born baby that breastfeeds and weaning food is slowly introduce after a given period of time, there is a distinct possibility that this expected scenario did not happen here. Death of the mother, sickness of the child or the mother, breastfeeding from a different female, even

contamination of the sample can result in these unexpected values. Unfortunately, there is not enough data to pinpoint one explanation for individual E-30.

For five samples where both oxygen and carbon isotopic values are increased (E-36A, E-35, E-21, E-6, E-29), solid food supplementation can be considered (Figure 15 and Figure 20). Although this argument cannot be ruled out, it should be noted that the range of variation for the carbon isotopic values is rather small, with differences mostly lower than 1‰ which could be attributed to other factors like fractionation of carbon isotopes in breast milk synthesis or during lactation, or simply the uncertainties of the stable isotope method.

Lastly, exclusive breastfeeding is indicated for individuals EE and E-39 from both carbon and oxygen signatures (Figure 15 and Figure 20).

## 5.4 Comparison with other studies

Comparing the results of this study with isotopic data from preceding studies on BWPs in Cuba is challenging. The two studies that provide information in this regard (Buhay et al. 2013; Chinique de Armas et al. 2017) include the isotopic examination of carbon and nitrogen signatures from bone collagen samples. This makes both the comparison of patterns of isotopic variation and the direct comparison of the isotopic values infeasible. However, these studies succeed to estimate relative ages of weaning. More specifically, evidence of supplementation using terrestrial plants was observed in Canimar Abajo with an estimation of a weaning age before 16±6 months of age (Buhay et al. 2013). Application of Bayesian models on the same population a few years later revealed that the process of weaning was completed by the age of three in Canimar Abajo. In the present study there was not enough data to support a weaning age estimation. However, there are signs of possible early supplementation that are mostly observed during the mineralization of the first permanent molar, which would mean the start of weaning somewhere after the fifth month of life.

An interesting case for isotopic data comparison comes from the Kaminaljuya population in Guatemala (Wright and Schwarcz 1998), which is culturally very close to that in the Caribbean. This is the only study to date which has used

subsequent enamel samples from the same individual, studying carbon and oxygen isotopic values. The basic difference with the present study is that Wright and Schwarcz used different teeth (premolars and first and third permanent molars) that mineralize later in life than the teeth groups analyzed here. Their results included variable patterns of isotopic changes which is also observed in the present study. They detected elevated $\delta^{13}C$ and depleted $\delta^{18}O$ signatures in older ages with nonsynchronous shifts occurring to carbon and oxygen isotopic values. They attributed these nonsynchronous shifts to extended complementary feeding practiced by the mothers.

The fact that the two studies which have sampled the same individuals multiple times (Wright and Schwarcz 1998 and the present one) have both led to variable isotopic patterns is of a great interest. It indicates that a lot of factors can play a significant role leading to different BWPs for each individual. This variability of the isotopic signatures in different periods of life for each individual introduces uncertainties and have an impact on the kind of conclusions that can be drawn in BWP research. The lack of specific patterns in isotopic shifts through the years can introduce biases when comparing data from different age groups, and restrictions in the method itself and the way it is applied. Despite the significant number of factors and contextual limitations that should always be part of the interpretations, there are also technical biases that can be confronted by alternative sampling procedures which might eventually lead to stronger conclusions.

## 5.5 Limitations

There are both contextual and methodological limitations that can affect the study of a past population. The majority of the time there are difficulties in characterizing ancient subsistence regimes and interpreting results from archaeological analyses. It is important to consider potential biases that may relate to the sample, the context or the chosen method of approach. For instance, in the present study, the variability of the intra-individual patterns of the oxygen isotopic values can indicate that there are factors which may affect the increase or decrease of the isotopic ratios in different periods of life. These factors can be individual (rhythm of growth, illness); cultural (practicing of breastfeeding or not, starting age of weaning or age of breastfeeding cessation); and/or methodological (error among how the teeth were sampled, sample size). These limitations will be further examined in the following paragraphs to examine potential biases.

### 5.5.1 Contextual implications

Rhythm of growth differs among individuals and this also translates to variation of teeth development and eruption (AlQahtani et al. 2010; Ubelaker 1989). Therefore, this fact can influence the time of enamel formation among individuals, which can lead to biases when specific time frames are under study. This study focuses on broader time intervals, rather than specific ones. The formation of the enamel for the three tooth categories occurs in the first few years of life (post-conception). The main time intervals used in this study are focused on the periods prior to and after birth. These intervals reflect the periods before and after breastfeeding and can allow us to observe significant and patterned alterations. Consequently, the variation in the formation of the enamel can possibly interfere with the results, but probably not to a degree that it can be considered as a major bias.

The presence of pathological signs and disease could suggest a potential source of bias in the final interpretations. A sick child might not receive the same attention from the parents and thus breastfeeding might be terminated earlier or might not start at all. This can lead to different isotopic ratios due to differential intake of water. In the anthropological study of the present population (Ruiz and Beatón 2000, 2) there were some pathological indicators. Furthermore, most of the

subadults that were sampled were infants under the age of three, an age which is in correlation with the expected mortuary rates of a population in general.

### 5.5.2 Sample size

One of the biggest limitations which osteoarchaeologists have to face is small sample sizes. In excavations there are rarely large numbers of skeletons recovered, especially with the all the necessary skeletal parts available. Also, this number can be influenced by the conditions of preservation or by the loss of skeletal material during excavation. Differential preservation of bone and teeth restricts the number of samples available to include in a study. In BWP studies the presence of subadult skeletons is crucial since they are considerably more likely to still have deciduous teeth in occlusion, which as demonstrated in this study give valuable evidence of the child's first years of life. There is also the case that the deciduous teeth themselves will not be distinguished and thus not recovered during the excavation, losing valuable evidence.

The size of the sample can interfere with the statistical analysis and the real-life representation of the population. The main drawback of a small sample size is the limited amount of data gained (regardless of the technique applied), which translates to statistical results that will most likely will lack accuracy. This is the most important factor of error that should be taken into account, since more conclusive generalizations should be avoided.

In the present experiment sample size was small (n = 34), mostly due to the lack of availability in deciduous teeth, especially incisors. Therefore, even though permanent first molars were sampled from 34 different individuals, it was only possible to sample deciduous teeth from nine individuals . The sampling of a tooth triplet was even more challenging and resulted in a sample size of five. This fact makes the interpretations more susceptible to bias and gives a greater possibility of error to the statistical analysis, where the p-value that is used depends on sample size. A sample consisting of more individuals makes the identification of statistically significant differences easier. It has been shown that if the sample is smaller than 20-30+ data points, the power to detect statistical differences decreases (Field 2009).

### 5.5.3 Methodological limitations

In isotope analysis, regression equations are used to predict the values of the drinking water ($\delta^{18}O$ in this study). It has been shown in recent years from various studies that a small error of approximately 1.5‰ is likely to present after the calibration of the data (Daux et al. 2008, 1138; Pollard et al. 2011, 499). In archaeology, the goal is not to predict the exact $\delta^{18}O$ values but to compare oxygen isotopic signatures among individuals and trace changes that can be informative. Using comparative isotope analysis, the typical error of the measurements ranges from 0.1‰ to 0.4‰ (Britton et al. 2015, 228), a margin much lower and more suitable for analysis. As it was described earlier, in this study fluctuations over 0.5‰ are considered changes important for interpretation. This way potential error due to measurements is considered negligible.

It is important to note that during the sampling procedure, enamel was extracted from different positions of a surface that represents the full mineralization period of every tooth, which can span several months or even years (AlQahtani et al. 2010, 485). During sampling, effort was made to target the same area of the crown for each tooth group. However, due to the bad preservation of much of the teeth and the fact that a thicker layer of enamel is deposited closer to the midpoint of the crown (middle point from the occlusal surface to the cervicoenamel junction), there can be a bias towards consumption which occurred in somewhat different periods of life among the individuals. For instance, the first permanent molars mineralize from birth (a time period which will be represented by enamel depositions near the cusps) and continues after the second year of age (a time period which will be represented by enamel deposition towards the end of the crown and the beginning of the root). Sampling different areas of the crown according to availability can show results from different life stages, leading to potential biases. For this experiment deciduous incisors and canines were sampled on the upper labial part of the crown towards the occlusal surface, deciduous molars were sampled closer to the root line, and first permanent molars were sampled on the middle crown (midpoint).

Lastly, the osteological paradox should be considered in every osteoarchaeological research. This phenomenon describes the biases introduced in the study due to the representation of exclusively dead individuals. In this way the

sample does not reflect the objective heterogeneity of the population and any reconstruction which is created will not be an equal reflection of the living population (Wood et al. 1992). Breastfeeding and weaning practices are highly related to the underlying pathology of the subadult individuals, since the isotopic signatures will show signals of ill children who did not survive into adulthood. As mentioned earlier these children may have received different treatment and went through different processes regarding their diet. From the other hand they could have died suddenly, and this would not have been translated in differences on BWPs. Including samples from adults in the study can be an acceptable solution in an attempt to overcome the osteological paradox. Other studies have mitigated these effects by sampling from bones with limited turnover like the petrous bone (Jørkov et al. 2009), or sequential sampling of bone fractions formed in different ages (using the metaphyses and diaphyses of long bones) (Waters-Rist et al. 2010) with limited success due to the phenomenon of bone remodeling.

## 5.6 Further research

BWPs is a relatively recent field that has started to build its foundations and explore new approaches. After the review of the technological limitations of isotope analysis application, together with the contextual boundaries of the archaeological samples, it is apparent that there is always a need for the adoption of new potential avenues that may contribute to the improvement of the results in future studies. There are different ways to approach these problems, and they will be further discussed here.

### 5.6.1 Introduction of a broader teeth sample

The interpretations in the present study are limited to the first two years of the infant's life. For further insights into how the isotopic values of carbon and oxygen vary after the start of weaning, when the introduction of liquid and solid food is taking place and breastfeeding moves towards cessation, the sampling of teeth that develop in later years can be highly informative. Studies have shown that additional samples of premolars (PM) and third molars ($M_3$), which are teeth that mineralize between three to seven and nine to twelve years of age respectively, can give valuable supplementary data (Wright and Schwarcz 1998,1999). Analyses of these teeth will further highlight the processes that happen during weaning practices and at the end of breastfeeding (White et al. 2004, 240).

Concerning carbon isotope analysis, it was expected that $\delta^{13}C$ values in $M_1$s would be enriched, since more supplementary nutrients are introduced to the child during the weaning period. Furthermore, the milk fats intake, which is likely responsible for lower $\delta^{13}C$ values, would be reduced. Therefore, the dental enamel $M_1$ $\delta^{13}C$ ratios should become higher towards the mother's diet, and thus may resemble the daily consumption of adults within the population (Wright and Schwarcz 1998, 12). Moreover, regarding $\delta^{18}O$ values after the enrichment that is expected during breastfeeding, the study of $M_2$ and $M_3$ will give valuable information for the changes that are taking place after the child's second year of life. Based on former studies, a decline of these values would indicate the start of weaning and a potential increase would support the continuation of breastfeeding (Roberts et al. 1988, 625-627; Wright and Schwarcz 1998, 3).

This kind of evidence can give indications of changing patterns after the period under study here, which will help even more with a distinction of a general trend in the isotopic values, potentially raising new questions about the results of the present research. Results of this kind can better visualize changes that happen during childhood, giving a more holistic picture of the fluctuation of carbon and oxygen isotope values. Furthermore, this would contribute to more accurate interpretations regarding the certainty of the introduction of weaning food and would help track changes brought by breastfeeding. In addition, by following the individual until adulthood, biases like the pathological state of the child that led to its death will be eliminated, as only individuals that survived childhood would be examined.

### 5.6.2 Incorporation of other isotopes

There is not as much research conducted in the BWPs field using particularly oxygen isotopic signatures as there is for nitrogen and carbon, although this study shows that the variation of $\delta^{18}O$ ratios can be highly informative for different aspects of BWPs and shed light on questions that cannot be so easily answered using other elements. However, the differentiation of the $\delta^{18}O$ values can be explored further in relation to and in comparison with the changes of other isotopes. In this study, carbon was used as a second supplementary element, but the incorporation of other elements in future studies of the sample can aid with the interpretation of oxygen isotopic values and with the reconstruction of weaning processes. These inquiries will add valuable information about different aspects of variation during supplementation that were not addressed in this study. Furthermore, the utilization of multiple elements is preferred when there is a possibility that consumption might differ among individuals, especially in studies like this with limited information provided.

Therefore, it is the suggestion of the author that nitrogen would be an informative addition since it has already been a valuable source of evidence for BWPs and has been studied extensively (Katzenberg et al. 1996, 188; Tsutaya and Yoneda 2015, 14). Further research of the same samples in combination with nitrogen isotope analysis can give supplementary data for the same individuals regarding their age of weaning. Nitrogen can be used to examine the protein intake using the trophic effect, and potentially pinpoint clearer reasons of the variation

observed in the present experiment. This way, the comparison of $\delta^{18}O$ values with $\delta^{15}N$ signatures can give insights for the intensity and duration of breastfeeding. For example, results from nitrogen isotopic signatures can show evidence of weaning food introduction through the consumption of different proteins where water intake would suggest that the infant is still only breastfeeding. The mechanisms which lead to the resulting isotopic signatures are different for oxygen, carbon and nitrogen, a fact that make these systems independent and thus informative. Together, data from carbon, nitrogen and oxygen will give information about different aspects of the child's diet. This data will contribute to a more accurate and holistic reconstruction.

Additionally, the possibility of spatial mobility of the mother prior to pregnancy can be examined with the incorporation of strontium (Sr) isotope analysis. Different provenance will also suggest different cultural backgrounds which may be responsible for the variation observed in some of the results in this experiment. As discussed previously (section 3.1.1, page 33), there are different theories for the provenance of the Archaic people on the island, and the multicultural aspect of the island's population was also presented by recent dietary studies (Chinique de Armas et al. 2015; Smith et al. 2018, 102-105). The utilization of Sr isotopic values can potentially provide information through the examination of the origin of the individuals included in the study, excluding in this way biases in the results that are related to differential provenance. For example, individuals can start with considerably different values as fetuses and approximate the rest of the individuals later (migration) or patterns of values may differ due to variable age of weaning (cultural variation).

### 5.6.3 Technical alternative approaches

It is also important to mention technological improvements that can be employed in future analysis. The field of isotope analysis has been moving forward rapidly in the last decades and continues to bring more knowledge and new advancements in analytical techniques. These techniques include those that improve the resolution of the results using smaller quantities of sampled material, or those that aid more accurate sampling. For instance, regarding teeth, more thorough sampling strategies which pay closer attention to the specific time periods represented in the sample can be both informative and minimize comparison biases. This can be achieved by more careful sampling of specific layers that form at

specific time periods. The consistency of the same sampling strategy applied for all samples can lead to results of higher resolution.

A finer resolution of seasonality effects can possibly be accomplished by the study of sequential samples of the same tooth, following its pattern of growth. Serial samples of the whole tooth has the potential to give valuable information about the local seasonal baseline. Human teeth form in layers, whose patterns are well-studied and defined (AlQahtani et al. 2010; Beaumont et al. 2013; Czermak et al. 2018). Seasonal differences can be reflected on human enamel and dentine which are deposited by layers during growth.

At the same time, it is becoming increasily more clear that as the knowledge in different fields increases, multidisciplinary approaches should be always considered and applied when possible. Presently, collaborations between physical anthropology, human ecology, archaeology and paleoanthropology are proven to be fruitful and promising, since BWP reconstructions can result in important insights for all fields. For instance, geochemical techniques and trace elements can provide objective evidence about modern human weaning practices. These inquiries, in turn, can contribute to archaeological research by shedding light on key biological processes that affect isotopic signatures.

# 6 CONCLUSIONS

The isotopic study of dental enamel from sequential samples of teeth was the focus of this study, providing the unique opportunity to examine even earlier life periods than previous studies by utilizing deciduous teeth. A combination of carbon and oxygen isotopic signatures was chosen in order to track different kinds of changes in the feeding practices of infants in the archaic Cuban population of Cueva del Infierno. The study concentrated on the isotopic variability occurring during the first two years of life. Three teeth were chosen to aid the objectives of this study: the deciduous incisors, the deciduous molars and the first permanent molars. These teeth were selected as they reflect the sequential periods of life *in utero*, around birth and the first year(s) after birth, respectively. This way, patterns in the isotopic signatures of young children can be traced, demonstrating trends and differences of the breastfeeding and weaning practices adopted by the mothers of the population under study, and potentially providing evidence about their relationships with their newborns.

Despite the small sample size, the application of isotope analysis in samples of dental enamel from the infants under study yielded some informative results that were presented in Chapter 4. The main objective of the present study has been met with more detailed interpretations presented in the discussion chapter (Chapter 5). In this present chapter, the most significant results and interpretations will be summarized, with the aim of answering the research questions posed in the introduction of this thesis.

**Isotopic patterns and their relation**

The main question of this study was whether or not patterns of variation that follow the dietary changes of infants exist, and if these can be traced in stable carbon and oxygen isotopic signatures. The results from the multi-sampled individuals in this study do not show a universal pattern of change for either carbon or oxygen ratios. The changes are more complex and unique for every individual, with fluctuations occurring in different forms, complicating and discouraging the possibility to make general interpretations

The variability of the patterns, even in a small sample like that of this research, can work as an indicator supporting the timing of weaning varies for each individual, with the values for each chemical element changing independently. The inconsistency of the values with increased age among children indicates a complexity that occurs when more precise and earlier in life interpretations are attempted. This results in the conclusion that there is not one trend or one theory that fits all, but there are multiple factors to take into account. The time of weaning varies among cultures, and social and/or individual differences can account for the differences observed in the isotopic ratios here. Alterations can also be attributed to potential variable maternal investment due to the underlying pathology of the infants. Although other possibilities of fluctuations were also considered, early supplementation and differential maternal investment due to a diseased child are considered the most probable. Initial stages of the weaning process were observed in both carbon and oxygen isotopic ratios, supporting the idea that supplementation was starting during the mineralization of the permanent first molars in Cueva del Infierno, and thus during the first 2.0-2.5 years after birth. Alternatively, similar variability in the isotopic ratios can be expected when different BWPs are adopted by the mother when the infant exposes underlying pathological signs.

To date, data from different age groups and different individuals have been compared in order to draw conclusions about the practice of breastfeeding and weaning in antiquity. This study has shown that different individuals have different patterns of isotopic shifts, a fact that can introduce uncertainties regarding the way that conclusion are drawn. Different factors can have different impacts in isotopic shifts that may be expected.

**Detection of liquid or solid supplementation**

Nutrient supplementation affects carbon and oxygen values in a different way, since the isotopic ratios of each element reflect different aspects of food intake: solid and liquid, respectively. There are some individuals that show signs of possible early supplementation, and these individuals were further examined in order to detect the different kinds of supplementation. The $\delta^{13}C$ ratios of E-30 showed the isotopic pattern expected during breastfeeding, while its $\delta^{18}O$ values indicated shifts related to weaning. Liquid supplementation can be speculated but

there was not enough data to exclude other possible reasons for this variation. Furthermore, five individuals appeared to have less negative isotopic values for both chemical elements during the mineralization of $M_1$, which can be evidence of solid supplementation. However, the isotopic shift is not significant enough to make such a strong argument . In this study, the lack of a sufficient amount of evidence to support liquid or solid supplementation indicates the necessity to incorporate different teeth groups and chemical elements in isotope analysis. This can lead to more nuanced insights in BWPs research, shedding light on the specifics of early supplementation in ancient cultures.

**Subsistence economy**

This thesis also intended to place the population into context in relation to other archaic populations previously studied in Cuba. Using the results from the carbon isotope analysis, the subsistence economy of the people of Cueva del Infierno was found to be mainly focused on $C_3$ plant consumption.

**Limitations of the study**

One of the main limitations of this study was the small sample size. The fact that this factor might have played an important role in the lack of isotopic patterns is recognized. There is a possibility that results may differ when greater numbers of individuals are studied. Although a larger sample size can be challenging for osteoarchaeological samples, performing more similar studies that utilize multiple samples from the same individuals, even with small samples, can aid to overcome such limitations, providing patterns of change from different populations.

Furthermore, the incorporation of nitrogen isotopic values could have been of a great help, tracking changes in the protein intake of the infants under study. Additionally, the sampling of teeth that grow later in life, wherever available, would also aid with further interpretations. Specifically, this would provide more accurate suggestions regarding the influence of breast milk to the isotopic signatures measured. Both alternative approaches were unfortunately difficult to accomplish due to low budget and time constraints.

**The importance of the study**

This work sets the fundamentals for further research in the field of breastfeeding reconstruction in Cuban subadults, and proposes a new approach for investigating biases and interpretations using carbon and oxygen isotopic ratios of multi-sampled individuals. As a starting point for future studies in the field of BWPs within the Caribbean in general, this study adds to the already acquired knowledge for these ancient populations and expands the boundaries of what can be achieved using the right sampling strategy when applying stable isotope analysis to osteoarchaeological material.

# 7 ABSTRACT

The present study is an attempt to reconstruct breastfeeding and weaning practices utilizing isotope analysis of human dental enamel. Nutrition during early childhood is directly related to the general health of a population. Studying the infant feeding processes in past populations is important for many scientific disciplines such as paleoanthropology, human ecology and primatology, shedding light on the evolution and reasoning that follows these practices. The purpose here is to explore patterns of change in the isotopic values before and after birth, collecting dental isotopic data that reflect the first two years of age. For this reason, infants from the archaic population of Cueva del Infierno in Cuba were multi-sampled for a triplet of teeth that includes a deciduous incisor, a deciduous molar and one permanent first molar. Another objective of the study is to place the population in context within Cuba and the Caribbean in general. Here, the results of carbon and oxygen isotope analyses reported from enamel sequential tooth samples indicate a $C_3$ plant-based diet for the population under study. Moreover, complex patterns of fluctuations are revealed and are attributed to early signs of supplementation or differential maternal investment due to possible underlying neonatal diseases/disorders. Furthermore, the detection and distinction of liquid supplementation is apparent for one of the individuals. Both practical and cultural limitations that may have influenced the outcome of the analysis are also thoroughly discussed. The isotopic analysis of the archaeological material in this study adds new evidence to the already acquired knowledge in regard to breastfeeding and weaning practices in the Caribbean. The application of similar practices for different ancient populations is recommended in order to expand the boundaries of isotope analysis application in osteoarchaeological material, offering a great opportunity for methodological and interpretational improvement.

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