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MALARIA IN THE PREHISTORIC CARIBBEAN: THE HUNT FOR HEMOZOIN

By

Mallory D. Cox  
B.A., University of Louisville, 2015

A Thesis  
Submitted to the Faculty of the  
College of Arts and Sciences of the University of Louisville  
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for the Degree of

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Department of Anthropology  
University of Louisville  
Louisville, Kentucky

May 2018



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A Thesis Approved on

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April 23, 2018

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## DEDICATION

This thesis is dedicated to my sweet, loving parents Michael Terry Cox and Deborah J. Trail Cox. Thank you for raising me to be fearless and to live my life through faith. You gave me the will, and He is making a way.

## ABSTRACT

### MALARIA IN THE PREHISTORIC CARIBBEAN: THE HUNT FOR HEMOZOIN

Mallory D. Cox

April 23, 2018

With the increase in resistance to anti-malarials and global warming trends expanding the habitation range of the mosquito vector, research highlighting the biogeographical contexts of infected populations is critical to understanding epidemiological patterns. A bioarchaeological approach to epidemiology can shed light on previous disease patterns and aid in the prediction of future outbreaks of diseases like malaria. Currently, there is no direct evidence of malaria in the Americas prior to European contact; however, skeletal, archaeological, paleoenvironmental, historic, and ethnohistorical evidence strongly suggest the presence of *Plasmodium spp.* malaria in indigenous Caribbean skeletal remains held in the Yale Peabody Museum of Natural History's (YPMNH) Caribbean Collection. Yale's collection is well preserved and represents indigenous populations inhabiting the Greater and Lesser Antilles from 300 BC-AD 600 and AD 1200-1500. Moreover, some individuals in this collection demonstrate healed or healing cribra orbitalia and porotic hyperostosis lesions on the cranium. One explanation for these anemia-related skeletal markers could be that they are the result of chronic hemolytic anemia, an adaptive response to malaria.



Hemozoin, an insoluble biomarker produced by all species of *Plasmodium*, shows promise in identifying malaria infections in blood samples of living individuals as well as in ancient skeletal remains. I utilized Matrix Assisted Laser Desorption Ionization Time of flight Mass Spectrometry (MALDI tof MS), Attenuated Total Reflectance- Fourier Transform Infrared Spectroscopy (ATR-FTIR), and Scanning Electron Microscopy (SEM) to identify hemozoin in indigenous Caribbean skeletal remains. The identification of *Plasmodium spp.* hemozoin crystals in this skeletal collection points to the presence of malaria in the Americas as early as AD 1000. These data will aid in the generation of a more complete epidemiological curve for *Plasmodium spp.*, enhance our understanding of the early spread of malaria, and contribute to biogeographical studies on European contact with indigenous populations.

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## CHAPTER 1: INTRODUCTION

Written records dating as far back as 5,000 years, including Greek, Babylonian, Chinese, Indian, Egyptian, and Assyrian sources, describe in great detail the intermittent fevers which are so characteristic of malaria infection (Neghina et al., 2010). These records demonstrate the degree to which malaria has burdened the health of humankind for millennia. This project aims to contribute to studies of malaria in both past and contemporary populations. Although traditionally thought to have come to the Americas with Europeans, recent phylogenetic evidence suggests the presence of *Plasmodium vivax* malaria in the Americas thousands of years before contact with Europeans (Carter, 2003). Malaria, unlike tuberculosis or syphilis, is an infectious disease not easily identified by bioarchaeologists and paleopathologists in human remains (Smith-Guzmán, 2015b). Furthermore, paleopathological investigations of malaria in New World populations are limited because of contemporary paradigms that exclude the possibility of malaria in the Americas prior to European contact. These paradigms suggest *P. vivax* malaria was transported to the Americas by Europeans and *P. falciparum* by Africans during the early colonial era (Mann, 2011). While it is very likely that each of these populations were carrying *Plasmodium spp*, this does not eliminate the possibility of an earlier introduction. This thesis investigates the possible presence of malaria in the Americas prior to the arrival of European colonists in the 1490s through multiple lines of evidence,

including skeletal, epidemiological, biogeographical, and ethnohistorical analyses. The results acquired using mass spectrometry, electron microscopy, and infrared spectroscopy reveal the presence of *Plasmodium spp.* hemozoin crystals in skeletal remains, pointing to the presence of malaria in the Caribbean as early as AD 1000.

In order to understand possible paleoepidemiological environments in precolumbian Circum-Caribbean contexts, a thorough understanding of the contemporary and historical epidemiology of malaria is necessary. Evidence in ethnohistorical records indicates a steady presence of malaria as early as the 16<sup>th</sup> century, only decades after European arrival in the Americas. The climate and ecology in the Circum-Caribbean supports *Anopheles* mosquitoes and *Plasmodium spp.* malaria today, but why do contemporary paradigms exclude the possibility of malaria in the Americas before European arrival? Does all the available paleoepidemiological evidence support this conclusion? What factors contribute to the introduction, incidence and transmission of malaria in human populations? Moreover, is there a way to test for the presence of malaria in antiquity? Written records constrain our understanding of the epidemiological curve for *Plasmodium spp.* in the Americas. However, a bioarchaeological approach permits us to extend our understanding of the paleoepidemiology of malaria by incorporating multiple lines of evidence to demonstrate the likelihood that *Plasmodium spp.* were present in the Americas prior to European contact.

Chapter 2 provides background on malaria as a human disease and discusses the current understanding of the geographical and temporal origins and evolution of *Plasmodium spp.* worldwide. I begin by discussing the current epidemiology of malaria, with a specific focus on the Americas. I review current research paradigms for the



geographic origins for *Plasmodium spp.*, specifically those that focus on the introduction of *Plasmodium spp.* to the Western hemisphere. I review the pathophysiological processes of malaria infections in the human host. In this chapter I discuss the relationship between malarial anemia and the etiologies of skeletal lesions attributed to anemia. I summarize the current state of the paleoepidemiology of malaria using human skeletal remains, and introduce the literature that suggests the biomarker hemozoin is a reliable indicator of malaria in skeletal remains.

Chapter 3 begins with a brief explanation of the current research paradigms describing the movement of people into and between the Caribbean islands in prehistoric time. I provide a chronology of cultural traditions identified by archaeologists on various islands beginning with the earliest inhabitants around 5,000 BC in the Greater Antilles (Cuba, Hispaniola, and Puerto Rico), leading up to the time period in which the skeletons sampled for this research have been associated including the ‘Saladoid’ cultural tradition (800 BC- AD 200) and the ‘Taino’ cultural tradition (AD 1200-contact). I address the ambiguity of the term Taino. Then, I recreate the paleoepidemiological environment of the Caribbean using ethnographic, ethnohistorical, and archaeological data. I provide available evidence for diet and subsistence in indigenous populations that suggests the anemia-related skeletal markers observed in these populations may have an alternate etiology, such as malaria infection and parasitic load more generally. Alternative interpretations of these anemia-related skeletal markers are acknowledged.

Chapter 4 provides detailed descriptions of the skeletal collections analyzed including a breakdown of the individuals sampled by estimated age and sex, reported by (Drew, 2009). The archaeological sites where the remains were recovered are provided in

a map as well as a table that includes details such as the exact long bone sampled and the methods of analysis employed for that sample. Explanations of the instrumentation used to analyze the skeletal samples are provided as well as references for hemozoin identification parameters, as cited in biomedical literature, and adopted for the purposes of this research. I also describe the sampling protocol, methods of sample extraction, sample preparation for each instrument, and analyses performed.

Chapter 5 presents the results of the analyses and a discussion on some of the caveats encountered throughout the investigation. I review the evidence for hemozoin and its precursors in this collection, and present images of hemozoin crystals observed in samples using scanning electron microscopy. I draw conclusions on the species of parasite that most likely produced the hemozoin crystals identified in the Yale Peabody Museum of Natural History's Caribbean Collection. This thesis concludes with a discussion on the implications of the findings as well as and possible directions for future research involving hemozoin identification in human skeletal remains.

## CHAPTER 2: THE ETIOLOGY AND EPIDEMIOLOGY OF MALARIA IN HUMANS

### *Epidemiology of Malaria*

Malaria is an infectious disease caused by a parasite of the *Plasmodium* genus and is transmitted between individuals through the bites of female mosquitoes of the genus *Anopheles* (NIH, 2016). Understanding the epidemiology of malaria, its distribution, and pathophysiology is crucial to the present study because these factors can be used to create a framework for investigating the presence of malaria in the past. For example, knowledge of the habitats of the mosquito vectors that transmit malaria is critical to recreating paleoepidemiological contexts which determine the likelihood of the presence of a vector-transmitted infectious disease such as malaria. It is also important to understand the processes occurring in the human body during an infection by the malaria parasite to understand which bones or part of the skeleton from which evidence of the parasite is most likely to be preserved. Each of these factors, as well as others that are relevant to the paleoepidemiology and identification of malaria in human populations will be addressed in this chapter.

There are five species of *Plasmodium* that currently infect humans: *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium ovale* (*P. ovale*), *Plasmodium malariae* (*P. malariae*), and *Plasmodium knowlesi* (*P. knowlesi*) (Smith-

Guzmán, 2015a). Malaria infections vary in their virulence, duration, and symptomology by species and strain (Setzer, 2010). The most common symptoms of malaria include intermittent fevers, violent chills, headaches, nausea, respiratory distress, severe anemia, sweats, and delirium (Masterson, 2014). Symptoms considered diagnostic of malaria are the intermittent fevers, which occur in cyclic patterns every 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale*, and every 72 hours in the case of *P. malariae* (NIH, 2017). These cycles correlate with the asexual stage of the parasite's lifecycle, which occurs in the red blood cells and is responsible for the symptoms associated with the illness (Masterson, 2014).

### ***Geographic Distribution of Malaria Infections.***

All five species of human malaria are a matter of global concern. *P. vivax* and *P. falciparum* are especially relevant to public health because they account for over 95% of the malaria infections worldwide (WHO, 2017). In the Americas, *P. vivax* accounts for 64% of all cases while 35% of malaria infections are attributed to *P. falciparum*, and the remaining 1% of cases are from *P. malariae* (WHO, 2017). However, *P. malariae* is commonly misdiagnosed using microscopy, especially in areas which are co-endemic for multiple species of *Plasmodium* (Barber, William, Grigg, Yeo, & Anstey, 2013). Recent work with populations in the Venezuelan Amazon demonstrated that 25% of individuals with *P. vivax* infections also contained *P. malariae* which was undetectable using the most common method of analyses- thick blood smear microscopy (Niño et al., 2016). The ability of *P. malariae* to remain in the host throughout their entire life, while carriers often remain asymptomatic, allows for this cryptic species to be overlooked (Rutledge et

al., 2017). Therefore, the presence of *P. malariae* in the Americas is certain, but the prevalence remains unclear (Bardach et al., 2015).

### ***Mosquito Vectors and Their Habitat.***

Understanding the habitat is crucial for understanding the potency of the vector and the likelihood of infection. For this research, knowledge of the vector habitat is important for understanding the potential of infection in precolumbian Caribbean populations. The mosquito vector is a critical factor in disease transmission because not only does malaria invade humans through the bites of infected *Anopheles* mosquitoes, but the sexual stage of the parasite's lifecycle takes place in the mosquito as well (MacKintosh, Beeson, & Marsh, 2004). *Anopheles* mosquitoes are present on every continent except Antarctica (CDC, 2015). There are over 460 *Anopheles* species (Afrane, Githeko, & Yan, 2012), and 30- 40 of them are efficient vectors for *Plasmodium* (WHO, 2017). The larvae of *Anopheles* mosquitoes are typically found in fresh or salt-water marshes, mangrove swamps, the edges of rivers and streams, grassy ditches, wet cultivation fields, and temporary or small pools of water (WHO, 2017). These larvae hatch between 2 days to 3 weeks after being laid onto the surface of the water, depending on the ambient temperature of the environment (NIH, 2016). The National Institute of Health (NIH, 2016) reports that *P. vivax* stops developing altogether when the temperature falls below 60 degrees Fahrenheit, which limits its seasonality and range in temperate regions (Paaijmans et al., 2010). In most tropical areas, reported cases of malaria infections increase during the rainy season (NIH, 2016). Climate change, deforestation, and urbanization all affect the rate of mosquito-transmitted infections such as malaria by changing the duration between feedings as well as the amount of time from

larval-to-adult development (Afrane et al., 2012). Andersen and Davis (2017) report an increase in the number of mosquito-borne diseases, such as malaria, in recent decades as the result of global warming temperatures.

### ***Origins of Plasmodium Spp. in Humans***

Sundararaman et al. (2016) highlight phylogenetic evidence for a transfer of *P. falciparum* malaria from gorillas to humans in Africa, likely within the past 10,000 years. Similarly, Liu et al. (2014) support the findings of Carter (2003) and deduce from phylogenetic analyses that *P. vivax* most likely represents a bottlenecked lineage that originated in Africa. According to Carter (2003), it is possible that all *P. vivax* are descended from parasites that infected the human populations in Sub-Saharan Africa and the Mediterranean between 100,000-20,000 years ago.

### ***Introduction of Plasmodium spp. to the Americas.***

Rodrigues et al. (2018) analyzed mitogenomes for *Plasmodium spp.* in human malarias worldwide to conclude that *P. falciparum* strains in the Americas were likely introduced in waves via African slaves throughout the 1500's. However, the introduction of *P. vivax* to the Americas remains unclear and highly disputed (Buery et al., 2017; Cornejo & Escalante, 2006; Loy et al., 2017). Mann (2011) has argued that *Plasmodium vivax* arrived in the New World with Europeans, based on evidence for *P. vivax* in England in the early 1600s. However, genetic analyses of *P. vivax* in the Americas today demonstrate the species has evolved to be sufficiently distinct from *P. vivax* in Asia and Africa to suggest an antiquity of thousands of years for *P. vivax* malaria in the New World (Carter, 2003). Carter (2003) proposes an initial spread from sub-Saharan Africa to southern Asia around 10,000 years ago, followed by a spread to the western Pacific

region, and by fast sea transport, across the Pacific Ocean to the Americas sometime in the last 5,000 years. Roullier (2013) highlights evidence of human contact between the Pacific Islands and South America 1,000 years ago. Rodrigues et al. (2018) conclude that *P. vivax* may have been introduced to the New World via Australasian populations prior to European contact. They argue this would explain the diversity seen in modern *P. vivax* strains in the Americas. Populations from distant regions of the globe were undoubtedly exchanging goods and ideas prior to European contact. It is likely that they were also exchanging infectious diseases, such as malaria.

### ***Plasmodium spp. in the Americas Today***

Nearly 50 years ago, attempts were made to eradicate malaria in the islands of the Caribbean (Rawlins, Hinds, & Rawlins, 2008). This was deemed successful in most islands with the exception of Haiti and the Dominican Republic. However, malaria is still a significant threat to all of the countries in the Caribbean, as demonstrated by the 2006/7 outbreak of over 340 cases of *P. falciparum* in Jamaica (Rawlins et al., 2008). In Peruvian Amazonia, 80% of all malarial infections are due to *P. vivax* species, and approximately 75% of those cases are asymptomatic low-grade parasitemias (Rovira-Vallbona et al., 2017). In Brazilian western Amazonia, symptomless infections of *P. vivax* and *P. falciparum* were identified as four to five times more likely than symptomatic infections (Alves et al., 2002). Asymptomatic low-grade parasitemias are characteristic of malaria-endemic environments such as those witnessed in Africa today (White, 2017). Furthermore, Sulzer et al. (1975) performed immunofluorescence testing for malaria antibodies on 123 indigenous people in southeast Peru. These tests revealed malaria antibodies in 109 individuals. Overall, *P. malariae* was identified in

97% of positive cases and *P. vivax* in 10%. *P. falciparum* was not identified in any individuals. Sulzer et al. (1975) concludes from these results that it might be possible for *P. vivax* and *P. malariae* to have been present in South America in precolumbian times. Bruce-Chwatt (1965) claimed it was probable that malaria was introduced to the Americas before European colonization.

***Secondary Host Populations for Plasmodium spp. in the Americas.***

Host transfers between humans and monkeys are a feature of malaria parasites though the direction of transfers in the Americas is debated (Escalante et al., 2005; Lalremruata et al., 2015; Rayner, 2015; Tazi & Ayala, 2011). *Plasmodium brasilianum*, a species of malaria found in New World monkeys, was recently identified in indigenous Yanomami populations living in remote regions of the Venezuelan Amazon (Lalremruata et al., 2015). Additionally, Madureira de Alverenga et al. (2018) found *Plasmodium simium*, another species infecting New World monkeys, in humans in Brazil. These trends suggest that host transfers between human *Plasmodium spp.* and monkeys in the Americas are common (Rayner, 2015). Moreover, the presence and prevalence of these secondary host populations in the Circum-Caribbean would put them in close living in the environments inhabited by indigenous groups for millennia. From an epidemiological perspective, this means New World monkeys could act as reservoirs for multiple species of *Plasmodium* that can infect humans (Tazi & Ayala, 2011). This factor lends further support to the likelihood that *Plasmodium spp.* could have transferred between human and non-human primate populations prior to European contact in the Americas.



## ***Pathophysiology of Malaria***

When investigating evidence of malaria via a biomarker produced in the human host, the pathophysiology of the infection is important to understanding where to look in the skeleton for evidence of the parasite. This research looks specifically at where in the body hemozoin is produced and sequestered. This is achieved by following the parasite from the initial point of entry in the human body via the bite of an infected *Anopheles* mosquito, through the following stages of the *Plasmodium spp.* lifecycle occurring in the human host. Within 30 minutes of being injected into the bloodstream of their human hosts, malaria sporozoites invade the liver to become protected from the immune system, and replicate profusely for 7-10 days (Masterson, 2014). At that time, they exit the liver and invade red blood cells to begin what is called the “intraerythrocytic stage” of infection, where millions of microbes feed on the protein components of the red blood cell and replicate further (Moxon, Grau, & Craig, 2011). The red blood cell is referred to by malariologists as the “epicenter of a genetic battle between the host and *Plasmodium* parasite”. Parasite-derived surface antigens effectively minimize the ability of the immune system to recognize infected red blood cells and instead allow for chronic infection (WHO, 2017). This is the stage where clinical symptoms set in, starting with extremely high fevers (Masterson, 2014). This is also the stage where the majority of hemozoin is produced by the *Plasmodium spp.* parasite, and therefore understanding these processes is critical to understanding the formation of hemozoin. Understanding the pathogenesis of malaria parasites in the human host informs sample selection protocols in order to increase the potential of identifying trace amounts of hemozoin in archaeological skeletal remains.

### ***The Relationship Between Malaria and Anemia.***

Certain infectious diseases, such as tuberculosis and syphilis, can be identified through specific skeletal lesions. Other infectious diseases, like malaria, do not produce unequivocal bony lesions, but result in conditions that may leave marks on skeletal tissue (Smith-Guzmán, 2015a). Alternately, the osteological paradox (Wood et al. 1992) could be applied to explain the absence of direct skeletal lesions for malaria in short-lived lethal malaria infections, such as those caused by *P. falciparum* cerebral malaria; the individual didn't live long enough for lesions to develop. Other species of *Plasmodium*, such as *P. vivax*, is known to cause long-term chronic infections and hemolytic anemia, but is associated with low mortality rates (White, 2017). Anemia can cause porotic hyperostosis (PH) (porous cranial vault) and cribra orbitalia (CO) (orbital roof lesions), which are identifiable in skeletal remains (Walker et al. 2009). However, the etiology of these lesions in human remains is not always clear, and they are typically attributed to multiple factors, and they may not be related (Rivera and Lahr 2017). Rivera and Lahr (2017) present evidence for a disassociation between CO and PH, while Stuart-Macadam (1992) argued for iron-deficiency anemia as the causative agent in porotic hyperostosis. Walker et al. (2009) have suggested that hemolytic and megaloblastic anemia may play a more dominant role in the development of porotic hyperostosis and cribra orbitalia. Additionally, Setzer (2014) notes that some inherited hemolytic anemia, such as sickle-cell anemia and thalassemia, are highly correlated with areas with a history of endemic malaria. Hence, although porotic hyperostosis and cribra orbitalia are not direct evidence of hemolytic anemia or malaria, these lesions occur in populations that experience frequent infections by *Plasmodium spp.* parasites.

### ***Malaria and Skeletal Markers for Anemia.***

Smith-Guzman (2015b) used modern reference samples to compare skeletal lesions in 98 individuals from Uganda, where malaria is holoendemic, to skeletal lesions in 106 individuals from a malaria-free zone. Smith-Guzman (2015) identified five skeletal lesions occurring more frequently in the malaria-endemic populations, including porotic hyperostosis, cribra orbitalia, porosity on the femoral and humeral necks, and porosity on the vertebral bodies. Additionally, in a study of 80 mummified individuals from Egypt, Rabino Massa et al. (2000) observed porotic hyperostosis in 92% of those individuals testing positive for *P. falciparum* malaria antigens. These data suggest that such lesions could be associated with malaria, but with more than one etiological explanation for cribra orbitalia and porotic hyperostosis, they can only serve as markers of anemia. The results of this thesis will inform studies on the relationship between anemia-related skeletal lesions with a potential for differential diagnosis or multiple etiologies, in skeletons of individuals with malaria. While beyond the scope of the current project, it should be noted that isotopic analysis to reconstruct diet would be a useful first step in attempts to understand dietary factors contributing to PH and CO in individuals presenting evidence of malaria in the skeleton, either in the form of *Plasmodium spp.* hemozoin, or *Plasmodium spp.* DNA.

### ***Malaria Detection in Paleopathology.***

According to Setzer (2014), paleopathologists rely upon three primary methods for interpreting malaria in the archaeological record: detection of ancient DNA (aDNA) from archaeological remains, immunological assays, and gross examination of human remains. Sallares and Gomzi (2003) successfully identified aDNA of the *P. falciparum*

malaria parasite within ancient human skeletal remains the 5<sup>th</sup> century AD in a Roman site in Italy. However, attempts to detect biomolecular signatures of the less virulent *P. vivax* in ancient human tissue have been unsuccessful (Smith-Guzmán, 2015b).

Immunological assays have proven successful in two cases. Rabino Massa et al. (2000) analyzed the mummified remains of 80 individuals from the site of Gebelen in Egypt dated to 3200 BC, finding malaria antibodies for *P. falciparum* in 42% of the samples. A study analyzed 155 tissue specimens (livers and spleens) from South American mummies (3000 BC to 600 BC) for evidence of malaria (*Plasmodium spp.*) using ELISA. From amongst these, 67% tested positive for *P. vivax* antibodies (Gerszten, Allison, & Maguire, 2012). This latter study is strong evidence for the *Plasmodium* parasite in the Americas as early as 3000 BC.

This research builds upon studies of paleopathology of malaria infections in skeletal remains through novel method of malaria identification via an insoluble biomarker: hemozoin. Hemozoin detection as a method of malaria identification, rather than aDNA or ELISA, is much less costly and requires less tissue for analysis. Moreover, hemozoin is an insoluble crystal that preserves well in settings such as the Caribbean, where the climate is not conducive to preservation of DNA. The preservation prospects of hemozoin might extend the possibility of detection of malarial infections in the Americas farther back in time, filling in gaps between the 5000-year-old infections detected in mummified populations (Gerszten et al., 2012) and those identified in the present study of Caribbean populations dating from AD 1000.

### ***The Role of Hemozoin in the Detection of Malaria***

Hemozoin is an insoluble, crystallized pigment, which sequesters and is preserved in specific bones of individuals infected with malaria (Setzer, 2014). Hemozoin is produced when the parasites invade red blood cells and digest hemoglobin, synthesizing amino acids and peptides from the globin while also releasing large amounts of free heme (Sun et al., 2016). The heme is toxic to the parasite because of its tendency for oxidation; therefore, it is converted in the parasite's digestive vacuole, i.e. "gut" into hemozoin, which is released into the bloodstream upon rupture of the red blood cell and subsequent invasion of other red blood cells by the parasite (Sun et al., 2016). Hemozoin is also deposited in the liver and spleen as a result of various processes including the first stage of the parasite's lifecycle in the human host, and processes initiated by macrophages of the immune system in an attempt to rid the blood and the body of waste and damaged red blood cells (Masterson, 2014)

Besides malaria, there are two other parasitic infections that produce hemozoin and are present in the Circum-Caribbean: schistosomiasis, (*Schistosoma mansoni*) and Chagas disease, (*Trypanosoma cruzi*) (Oliveira et al., 2005). Each species of hemozoin-producing parasite demonstrates uniquely-shaped crystals that can be differentiated using Scanning Electron Microscopy (SEM) (Noland et al., 2003). Hemozoin is therefore a 'biomarker' for malaria and other hematophagous organisms. Setzer (2014) argues that residual amounts of hemozoin may be detected in medullary cavities using physical, chemical, or histological methods.

### *Hemozoin as Evidence of Malaria in Caribbean Skeletal Remains*

Newly developed methods allow archeologists to identify direct evidence of malaria in ancient skeletal remains via the biomarker hemozoin (Inwood, 2017). This research employs and adapts these methods to inform studies on precolumbian populations in the Caribbean Islands, and in Puerto Rico more specifically. Individuals sampled from the Caribbean Collection at YPMNH present indicators of anemia in most of the crania, markers that could be caused by chronic or inherited hemolytic anemia. In the next chapter, I consider the cultural and epidemiological context of the Caribbean, demonstrating the presence of the vector in local environments, as well as dietary information that suggests iron-deficiency anemia would have been unlikely. Hence, the presence of such lesions, known to be adaptive responses to malaria whether in the form of chronic infections or as inherited traits, might indicate a malarious environment. Subsequent chapters deal with additional lines of evidence needed to support this initial hypothesis.

## CHAPTER 3: INDIGENOUS CARIBBEAN CULTURAL TRADITIONS: THE SALADOID AND THE TAINO

### *Indigenous Colonization of the Caribbean Islands*

I analyzed indigenous Caribbean skeletal remains to look for evidence of malaria in the Americas prior to contact with Europeans. Archaeological, paleopathological, ethnographic and ethnohistorical data strongly suggest the presence of *Plasmodium spp.* in antiquity. In this chapter I review archaeological, ethnographic, and ethnohistorical data to highlight evidence to support this hypothesis. I include considerations of diet and nutrition, anthropogenic landscape alterations, and cultural adaptations to mosquitoes as pests.

I begin with a review of the chronologies of human settlements in the Caribbean islands in order to contextualize the sample population in antiquity. Caribbean archaeologists refer to the first inhabitants of the Caribbean Islands belonging to one of two major 'Archaic' period cultural traditions: the 'Ortoiroid' who appear in the record around 5000 BC in Trinidad and Puerto Rico, and the 'Casimiroid' who began to occupy the coasts of Haiti and Cuba around 4000 BC (Keegan and Hofman, 2017). The Ortoiroid cultural tradition is believed to have migrated to the Caribbean from northwest Guyana, while the Casimiroid tradition originated in northwestern South America or Central America (Keegan and Hofman, 2017). The traditional periodization in Caribbean archaeology includes the Archaic period followed by the Ceramic period. However, Reid

(2014) has recently identified pottery production in the Archaic period in the islands as including a period of experimentation, where different pastes and decorative techniques were explored.

The Early Ceramic period (800-200 BC) in the Caribbean begins with waves of migrants of the Saladoid ceramic tradition. This tradition is found in abundance from Trinidad and Tobago to Puerto Rico beginning between 800 and 500 BC (Reid, 2014; Keegan and Hofman, 2017). The term ‘Saladoid,’ named after the Saladero site in Venezuela where they were originally discovered, refers to the highly formalized styles used in decorating ceramics, particularly white-on-red incised pottery (Keegan and Hofman, 2017).

In Puerto Rico, the focus of this study, the Saladoid tradition disappears around 500 AD and is followed by the Early Taino period (AD 600-AD 1200), and then the Classic Taino period (AD 1200-contact). The Classic Taino produced ceramics that occasionally featured red slip (Curet, 2005). In excavations performed by Rouse and Rainey in the 1930s and 1940s, contexts were relatively dated using these ceramic sequences. The human remains investigated in this research were recovered alongside material artifacts associated with the Saladoid and the Taino ceramic traditions (Drew, 2009).

### ***‘Taino’: the Complexity of a Term***

The term 'Taino' is under heavy scrutiny by Caribbean scholars who claim it masks the diversity of peoples and groups inhabiting the islands when Europeans arrived (Feliciano-Santos, 2017). Although the term ‘Taino’ is used by archaeologists to refer to



Caribbean Island populations in the past, regional differences in language, culture, and group identities were present (Curet, 2005). Evidence of cultural diversity among and within the islands is present in the writings of Columbus following his visits to the Bahamas in 1493. Columbus' records indicate that the peoples he encountered in the Bahamas greeted him with the word 'Taino', which translates into 'good' or 'nice' in the Taino language, but which Columbus began using as a cultural identifier. Those 'Taino' whom he met in the Bahamas described their neighbors on the island of Cuba as the 'Ciboney' and the 'Guanahatebey' (Keegan and Carlson, 2008). 'Lucayan' Taino refers to the cultural tradition of those peoples who began colonizing the Bahamas and the Turks and Caicos Islands around 600AD (Keegan and Carlson, 2008). 'Classic' Taino is the term used by archaeologists to describe the material culture of the peoples living on the islands of Puerto Rico, Haiti, Cuba, Jamaica and the Dominican Republic from AD 1200 onward (Keegan and Carlson 2008).

Each of these cultural groups, as well as others, has at one time or another been grouped into the designation 'Taino,' similar to distinct Native American tribes being referred to solely as 'Native Americans.' Furthermore, there are living populations who identify as Taino. In fact, the 2010 US census revealed that 9,399 people in Puerto Rico identified as Taino alone or in combination with other categories (Feliciano-Santos, 2017). Acknowledging the complexity of the term Taino and recognizing the ambiguity that may result from its use, it is beyond my experience, and the time constraints of a thesis, to propose alternatives lexicons. For this research the designations 'Saladoid' and 'Taino' are used as markers of populations, with Saladoid referring to individuals living

after 300 BC at archaeological sites in Puerto Rico and Taino referring to individuals inhabiting archaeological sites in Puerto Rico after AD 600 until contact.

### ***Navigating the Open Water: Networks of Exchange and Interaction***

The Taino, along with their Saladoid ancestors, and other Circum-Caribbean populations were accomplished maritime travelers. Canoes were dug out of a single log and used as a means of transportation from one island to the next using paddles instead of sails (Keegan and Hofman, 2017). Columbus observed many of these brightly colored canoes in special sheds on the beach in southern Cuba, and the largest canoes have been known to carry approximately ninety passengers (Keegan and Carlson, 2008). Ceramic, lithic, botanical and genomic data suggests the entire Orinoco River basin was a zone of intensive intercultural contact (Ramos, 2010). Additionally, recent analyses of ancient DNA from remains dating to about 1000AD recovered at a site in the Bahamas show a strong affinity between the indigenous Caribbean and modern South American populations, particularly the Yanomami from the Amazonian region (Nieves-Colon, 2014). At some of the earliest Archaic sites in Cuba, archaeologists have identified fruits native to Central America (Keegan and Carlson, 2008). The continuity in relatively long-distance voyaging between the islands and the mainland is indicated by the exchange of people, good, and ideas over millennia (Keegan and Hofman, 2017). As a result of the interconnectedness of the indigenous populations of Central America, the Caribbean, and northern South America, this research considers this region, the Circum-Caribbean, to be a paleodemographic unit.

### ***Diet and Subsistence.***

The Taino in Puerto Rico practiced a form of slash and-burn horticulture where they cleared a patch of forest to create a permanent field close to the main village

(Keegan and Hofman, 2017). In the field they built raised mounds of earth to cultivate root crops (Keegan and Carlson, 2008). This form of agriculture is referred to as ‘conuco’ (Keegan and Hofman, 2017). Spanish records indicate that the Lucayan Tainos cultivated as many as 80 different plants (Keegan and Carlson, 2008). These included staples like sweet potatoes and manioc, a tuber that is processed to remove poisonous juices, leaving a pulp that is dried and prepared into bread. The Tainos also planted coco yams, beans, gourds, corn, cotton, chili peppers, sweet and bitter manioc, papaya and guava (Keegan and Carlson, 2008). Animal protein came primarily from the coastal marine environment, including crabs, shellfish, and fish, though some land animals, such as iguana and hutia, were available in limited quantities (Keegan and Carlson, 2008).

Ethnohistorical records describe the Taino populations as highly skilled fishermen (Keegan and Carlson, 2008). In addition to many species of fish, the Taino were known to catch stingrays and sharks. Sharks’ teeth and the stingray spines were used for multiple things including to tip hunting spears and for fishing purposes, and also as materials for tools like drills, and decorative or symbolic items (Keegan and Carlson, 2008). The use of hooks, lines, nets, and bows and arrows are all fishing methods observed in the indigenous populations, according to Spanish records (Keegan and Carlson, 2008).

Human skeletal remains were recovered on Puerto Rico from coastal middens overwhelmingly composed of crabs and freshwater mollusk shells (Rouse, 1993). These archaeological data, along with skeletal observations showing little evidence for malnutrition or frailty, such as thin cortical bones or linear enamel hyperplasia, as reported by Drew (2009), suggest that these populations were not suffering from inadequate nutrition. The skeletal remains, including the dentition, from the island of

Puerto Rico in YPMNH's Caribbean Collection paint a picture of a subsistence system and lifestyle conducive to good health. While this sample is too small to be used to make inferences about the health of entire indigenous populations, this sample does suggest good 'overall' health for the majority of Saladoid and Taino individuals recovered at sites in Puerto Rico. The remainder of this chapter will be aimed at illustrating a paleoepidemiological context in the Circum-Caribbean region using available archaeological, ethnohistorical, and ethnographic data.

***Settlement Patterns and Anthropogenic Landscape Alterations: Any Relation to Incidence of Malaria?***

Taino coastal villages in the Bahamian Islands were composed of houses atop a sand dune with the ocean in front and a marshy area behind (Keegan and Carlson, 2008). These marshy areas are significant to this research because these small bodies of water are ideal microenvironments for *Anopheles* mosquitoes to complete their lifecycle (Barros & Honório, 2015). Additionally, Rivera-Collazo (2015) has found evidence in anthrosols that point to forest clearing in the Caribbean islands by indigenous populations as early as 3000 BC in Puerto Rico. Forest clearing today has been shown to directly correlate with an increase in the malaria vector *Anopheles spp.*; thus a high risk of malaria incidence has been associated with forest fringe environments where anthropogenic landscape alterations have occurred (Barros & Honório, 2015). In the Archaic period in the Caribbean, and following multiple waves of migrations to the islands throughout the Ceramic period, a change in indigenous settlement patterns that included forest clearing would have increased the mosquito population at a local level.

***Pesky 'Jejen' and 'Malaria Bark': Regional Evidence for Mosquito-borne Diseases.***

There is evidence for mosquitoes as a known pest throughout northern South America, especially along waterways such as the Orinoco River. The Orinoco River was well known by European and Spanish explorers in the 16th century for its giant mosquitoes, *Anopheles darlingi*, which came off the river in the evenings “in swarms so thick it was difficult not to inhale them” (Honigsbaum, 2002). The fast-flowing river, full of silt, provided the perfect breeding grounds for mosquito larvae of *Anopheles* mosquitoes, an effective malaria vector (Honigsbaum, 2002). ‘Jejen’, a Taino term for mosquitoes, occurs frequently in ethnographic narrative. These records, as collected and reported by Keegan and Carlson (2008), include Taino descriptions of mosquitoes as pests and cultural adaptations to deter mosquitoes. Notably, adaptations to an infectious environment are suggested in the use of a mosquito repellent which the Taino called ‘bija.’ The repellent includes a paste made of the ground seeds of achiote, fruit-bearing trees native to lowland South America that were brought to the Bahama archipelago thousands of years ago (Keegan and Carlson, 2008). Clearly, mosquitoes were a known pest throughout the Circum-Caribbean.

It is possible, as suggested by indigenous methods for treating fevers, that Saladoid and Taino populations associated increased intermittent fevers with mosquito-ridden environments. Some medical historians argue that Andean healers used the bark of the cinchona tree to treat ‘fevers’ for some unknown length of time before the Jesuits took it back to Europe in the mid-17<sup>th</sup> century (McNeill, 2010). Other historians argue indigenous peoples didn’t know of the uses of cinchona bark (Honigsbaum, 2002). However, ethnohistorical documents provide insight into the ways indigenous South American populations were treating fevers. For example, the journal records of a French

botanist Joseph de Jussieu, who was traveling in the Americas looking for cinchonas in the mid-1700's, describe how he fell seriously ill with fever while passing through a malarious region in Ecuador (Honigsbaum, 2002). While there, he encountered the 'Malacatos Indians,' who inhabited the region, and a local *cacique* offered to cure de Jussieu. The *cacique* went to the nearby mountains to fetch the fever bark which 'miraculously cured' the explorer (Honigsbaum, 2002).

The name 'fever bark' or 'malaria bark' as it referred to in other ethnohistorical records (McNeill, 2010), indexes the medicinal properties of the bark of the cinchona tree to treat malarial fevers. This tree grows naturally in Ecuador, Columbia, and Peru, often in remote parts of the eastern and northern Andes above 1,500 meters, but has also been discovered in Merida, an Andean town in northern Venezuela that is close to the coast (Honigsbaum, 2002). In the 17<sup>th</sup> century, the only place in the world in which this antimalarial plant was found growing was in these at high-elevation Andean environments. These zones extend into Northwestern Venezuela from which multiple indigenous migrations into the Greater Antilles are thought to have originated (Keegan and Carlson, 2017). The Puerto Rican Saladoid and Taino peoples were likely in contact with coastal populations in northern South America. Hence, it is likely that indigenous knowledge of the medicinal properties of cinchona tree bark for the treatment of malarial fevers was shared.

*Detecting Malaria in Antiquity in the Caribbean: The Promise of Hemozoin.*

The epidemiological landscape has been recreated using research conducted in archaeology, paleopathology, ethnohistory and ethnography. An epidemiological explanation for anemia-related skeletal markers, such as living in a malarious

environment, is preferred to a nutritional explanation. Ethnohistorical and paleopathological evidence indicate indigenous people did not suffer from inadequate iron sources in their diet. Therefore, it is unlikely that the anemia-related skeletal markers present in many Caribbean skeletal remains were the result of iron-deficiency anemia, especially in the absence of other skeletal markers indicative of inadequate nutrition. Given that an antiquity of *Plasmodium vivax* in the Americas is suggested by genomic data (Carter, 2003), and that the paleo-epidemiological landscape strongly supports the maintenance and transmission of malaria in the Circum-Caribbean region before the arrival of Europeans, an epidemiological explanation for these markers is proposed instead.

Recent investigations have demonstrated hemozoin can be preserved in skeletal remains for thousands of years (Inwood, 2017). If malaria was present in the Circum-Caribbean, the preserved skeletonized Saladoid and Taino populations would present evidence of hemozoin left in residual amounts in long bones.

## CHAPTER 4: MATERIALS AND METHODS

### *Materials*

The Yale Peabody Museum of Natural History's (YPMNH) Caribbean Collections are the product of excavations conducted in the 1930s and 1940s by Froelich Rainey and Irving Rouse (Drew, 2009). The Caribbean Collections include human remains representing 72 individuals with degrees of preservation ranging from excellent to poor (Drew, 2009). In addition to the human remains, this collection includes ceramics, worked bone, perforated shells and marine and terrestrial faunal remains (Drew, 2009). I chose to sample individuals from only the island of Puerto Rico because these remains are the most complete. Furthermore, the Puerto Rican subset represents eight sites including coastal and inland locations and includes occupations from both the Saladoid (300 BC-AD 600) and the Taino (AD 1200-contact) cultural traditions, according to ceramic associations reported by Rouse (1992), as cited in Drew (2009). These factors allows for the possibility of comparative analyses across multiple spatio-temporal contexts (Fig. 4.1). The remainder of the Caribbean Collection consists of individuals from the Bahamas, Cuba, Haiti, and Venezuela (Drew, 2009), but was not observed for this research. Individuals from the Puerto Rico subset were recorded as associated with the Saladoid cultural tradition if they were recovered interred with Saladoid style ceramics. Individuals were associated with the Taino if cranial modifications were observed, if the burial was from within a shell midden, or if interred with Boca Chica or Santa Elena ceramics (Drew, 2009). I examined the Puerto Rican subset of skeletons,



including 27 individuals (catalog numbers PA151-PA173), and recovered 32 samples from 20 individuals that presented appropriate bones (see Table 4.1).

Figure 4.1 Site Locations for Skeletal Remains Sampled from Puerto Rico



Map source: Author (2018); Cite locations as reported in Drew (2009)

Table 4.1 Summary of Analyses Conducted

#	YPM Catalog ID	Site	Sex	Age	Element	Crania	PH	CO	SEM	FTIR	MALDI
1	PA152 B2,I2	Toa Baja 2	M	Adult	Femur	-	NA	NA	<b>X*</b>	-	-
2	PA152 B3,I4	Toa Baja 2	M	Adult	Femur	-	NA	NA	-	-	-
3	*PA163 B2,IB	Monserate	M	12-14	Humerus	+	+	NA	<b>X*</b>	-	X
4	PA163 B2,IB	Monserate	M	12-14	Femur	+	+	NA	-	X	-
5	*PA161B1,IA	Collores 2	F	>25	Femur	+	+	+	<b>X*</b>	X	-
6	PA161B1,IA	Collores 2	F	>25	Femur	+	+	+	-	-	X
7	PA161 B1,IA	Collores 2	F	>25	Tibia	+	+	+	-	X	-
8	*PA164 B1	Canas 2	F	>45	Femur	-	NA	NA	-	-	X
9	PA164 B1	Canas 2	F	>45	Humerus	-	NA	NA	-	X	-
10	PA152 B3,I1	Toa Baja 2	M	>35	Humerus	-	NA	NA	-	X	-
11	*PA150 B1,I1	Cabo Rojo 11	M	Adult	Humerus	+	+	NA	-	X	X
12	*PA151 B1,I1	Toa Baja 2	F	>35	Femur	-	NA	NA	X	X	<b>X*</b>
13	PA151 B1,I1	Toa Baja 2	F	>35	Tibia	-	NA	NA	<b>X*</b>	X	-
14	*PA173 B1	Yauco 1	M	Adult	Tibia	+	+	+	-	<b>X*</b>	-
15	PA173 B1	Yauco 1	M	Adult	Femur	+	+	+	-	<b>X*</b>	-
16	PA173 B1	Yauco 1	M	Adult	Humerus	+	+	+	<b>X*</b>	X	X
16.5	PA173 B1	Yauco 1	M	Adult	Tibia	+	+	+	-	X	-
17	PA170	Santa Isabel	M	Adult	Femur	-	NA	NA	-	X	X
18	PA165 B1,I1	Unknown	NA	Adult	Humerus	-	NA	NA	-	X	-
19	*PA162 B2	Collores 2	NA	2.5 y.o.	Femur	-	NA	NA	X	-	-
20	PA162 B2	Collores 2	NA	2.5 y.o.	Femur	-	NA	NA	-	X	-
21	PA150 B1,I1	Cabo Rojo 11	M	Adult	Humerus	-	NA	NA	-	-	X

22	PA153 B2,I4	Toa Baja 2	NA	5 y.o.	Humerus	-	NA	NA	-	X	-
23	PA153 B2,I2	Toa Baja 2	M	Adult	Ulna	-	NA	NA	-	-	X
24	PA153 B2,I3	Toa Baja 2	NA	1 y.o.	Radius	-	NA	NA	-	-	X
25	PA163 B2,IA	Monserate	NA	Adult	Ulna	-	NA	NA	-	-	-
26	PA163 B2,IB	Monserate	M	12-14	Ulna	-	NA	NA	-	-	-
27	PA165	Unknown	NA	Child	Radius	-	NA	NA	-	-	-
28	PA165	Unknown	NA	6 y.o.	Ulna	-	NA	NA	-	X	-
29	PA166	Unknown	NA	Child	Radius	+	+	NA	X	-	-
30	PA166	Unknown	M	>50	Femur	-	NA	NA	-	-	X
31	PA167	Salinas 1	M	Adult	Ulna	-	NA	NA	-	-	-
32	PA161 B1,IA	Collores 2	F	>25	Femur	+	+	+	-	-	-

KEY:

M-Male  
F-Female  
Ind-Indeterminate  
Element- Bone sampled  
SEM- Scanning Electron  
Microscope  
FTIR- Fourier Transform Infrared  
Spectroscopy  
MALDI- Matrix Assisted Laser  
Desorption/Ionization time-of-flight  
Mass Spectrometry

PO-Porotic hyperostosis  
CO-Cribra Orbitalia  
(+)- Presence of lesion  
\*next to YPM Catalog ID indicates  
more than one sample taken for the  
individual  
(-) - Absence of lesion  
X\*- Results identifying hemozoin  
X- Analysis conducted  
NA-not available  
y.o. - years old

Table 4.2 FTIR Infrared Wavelengths for Hemozoin (Reference)

1206	1207	1208	1210	
1661	1662	1663	1664	1712

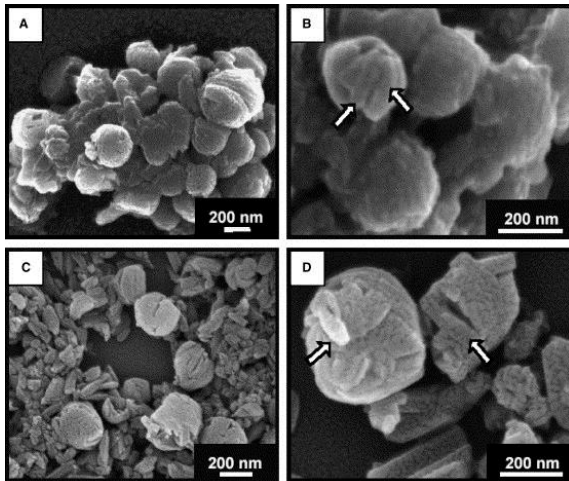
Source: Tempera et al. (2015), Lvova et al (2016)(Oliveira et al. (2007), Samson et al. (2012) Egan et al. (2001), Webster et al. (2009)

Table 4.3 Parameters\* for the Identification of *Plasmodium spp.* and *Schistosoma spp.* Hemozoin Crystals using SEM

<i>Plasmodium</i> Species	Size of Hz Crystals	Description of crystal
<i>P. Malariae</i>	200nm(wide)x 200nm(cross-section) x 600nm(length) and 100nm x 100nm x300nm	Smooth flat-faces
<i>P. Brasilianum</i>	75nm × 75nm × 400 nm	Very similar to malariae
<i>P. Ovale</i>	75 nm× 75nm×300–400nm	Squarish cross- sections
<i>P. Vivax</i>	50-100nm x 100-150nm x 300-500nm	Some crystals are very thin and wide creating rectangular cross-section
<i>Plasmodium spp.</i>	100nm x 100nm x 300-500nm (Hz crystal average)	
<i>Schistosoma spp.</i>	200 nm long bricks	Assembled into multi-crystalline spherical structures

\*Source: Noland et al. 2003 (*Plasmodium spp.*); Oliveira et al. 2005 (*Schistosoma mansoni*)

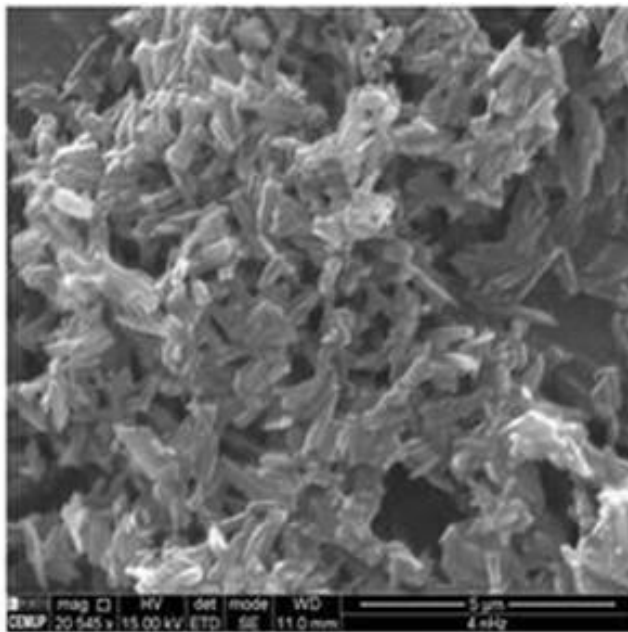
Figure 4.2 SEM Hemozoin Crystals from *Schistosoma spp.* and *Trypanosoma spp.* (Reference)



Field emission scanning electron microscopy of Hz crystals isolated from *Schistosoma mansoni*

Source: Oliveira et al. 2005

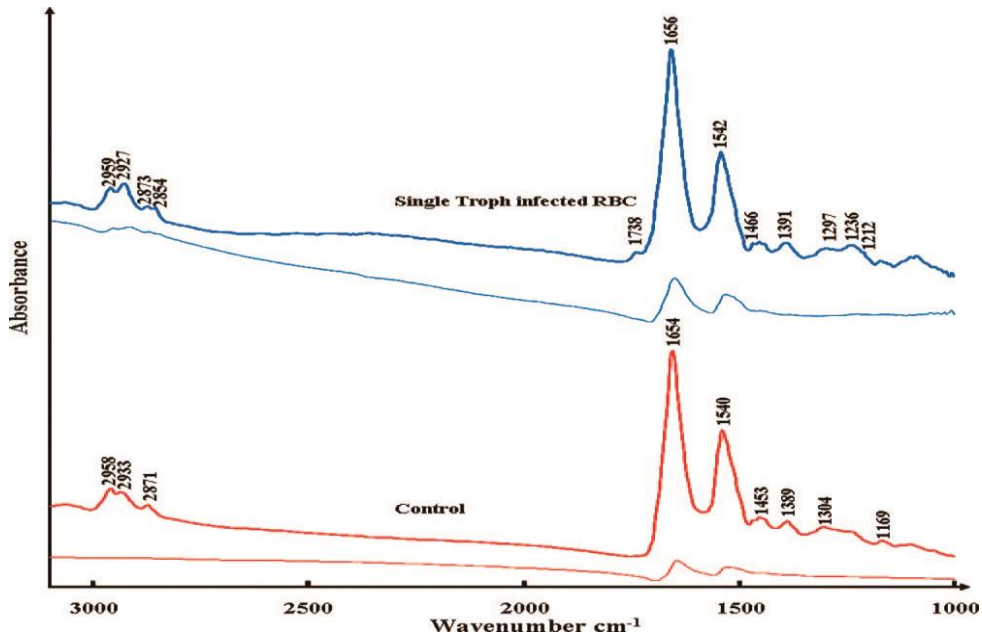
Figure 4.3 SEM Hemozoin Crystals from *Plasmodium falciparum* (Reference)



Scanning electron microscopy image of hemozoin purified from *P. falciparum* culture (20,000x magnification)

Source: Tempera et al. 2015

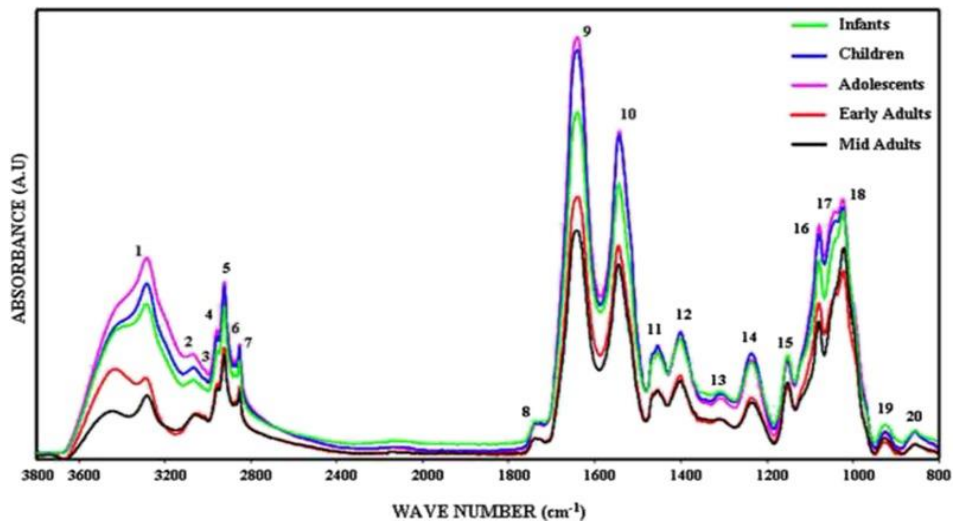
Figure 4.4 – ATR-FTIR Wavelengths Identifying Red Blood Cells (Reference)



Source: Webster et al. 2009

Mass spectra identifying wavelengths for red blood cells using ATR-FTIR

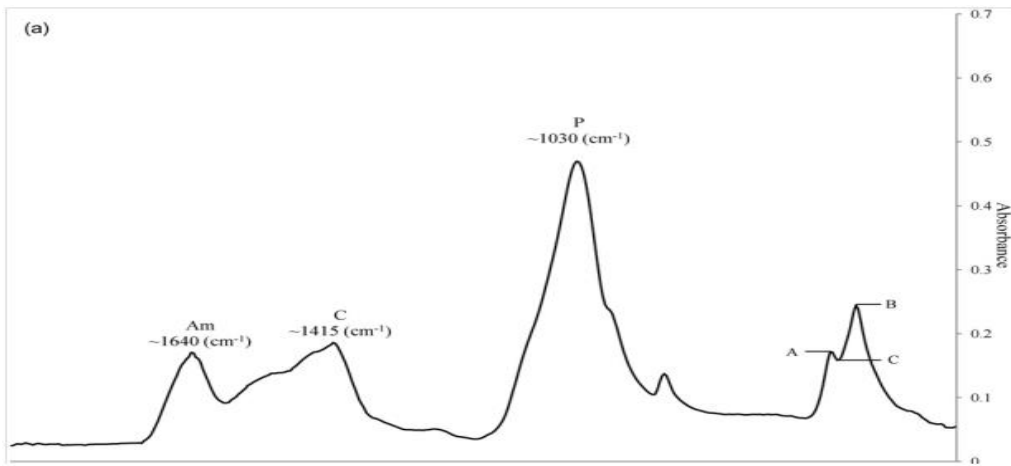
Figure 4.2 ATR-FTIR- Wavelengths Identifying Bone Marrow (Reference)



Source: Aksoy et al. 2014

Mass spectrum identifying wavelengths for bone marrow using ATR-FTIR

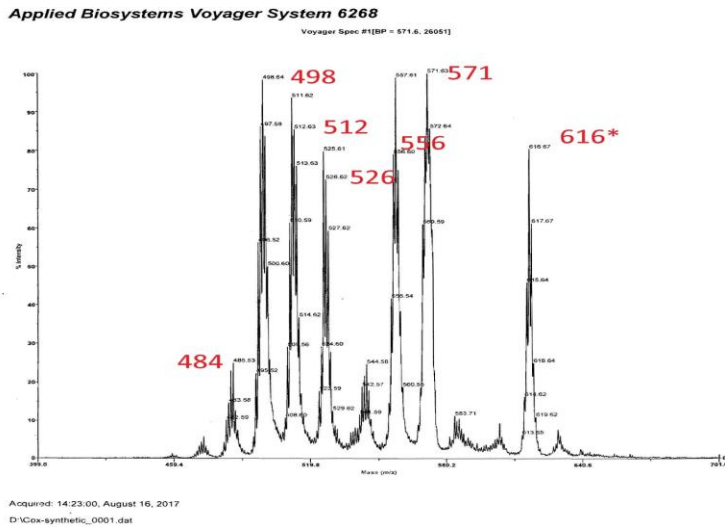
Figure 4.3 FTIR-ATR Wavelengths for Bone (Reference)



Source: Hollund et al. 2013

Mass spectrum identifying wavelengths for bone using ATR-FTIR

Figure 4.4 MALDI tof MS Results for Synthetic Hemozoin (Reference)



Source: Laposki (2017)

Mass spectrum of synthetic hemozoin demonstrating the presence of molecular ion 616\* and all fragmentary ions associated with hemozoin

## ***Methods***

### ***Protocol for selecting individuals for sampling.***

Guidelines for selecting individuals for sampling included the presence of two long bones per individual skeleton, ideally femora or humeri, and some portion of the cranium, with preference given to adults. Cranial fragments allow for further analysis of correlations between anemia-related skeletal markers, (i.e. cribra orbitalia and porotic hyperostosis), and those individuals in which hemozoin is identified. I planned to select adults preferentially based upon the premise that a longer life corresponds to more opportunities for infection, thereby increasing the chances for hemozoin production and sequestration. This would increase the likelihood of detection. Cranial fragments were present for 6 individuals (see Table 4.1) and each demonstrated evidence of porotic hyperostosis while 2/2 orbits demonstrated cribra orbitalia (see Figure 4.8.1 (A)).

In adults where the long bones present were hollow and the cancellous tissue was entirely absent from the medullary cavity, the likelihood of hemozoin preservation was highly diminished, hence long bones in this state of preservation were given a lower sampling priority. Similarly, some long bones were ‘fragmented’ but mostly complete, thus, it was impossible to access the medullary cavities with the sampling instruments utilized in this research.

### ***Hemozoin-like Material.***

For the purposes of this research I will use the terms ‘hemozoin-like material’ in reference to black pigmented granules with a crystalline surface macroscopically observable in sampled long bones (See Figure 4.8.2 (A,B). I observed optimal



preservation of this hemozoin-like material in long bones in which the proximal ends were complete and the distal end was fragmented. The fragmented end provided access to the medullary cavity for sampling instruments, and the complete proximal end protected the cancellous bone from taphonomic processes. Thus, femora and humeri with the distal end fragmented and the proximal end complete were prioritized over other long bones.

***Sampling Protocol (Step-by-Step).***

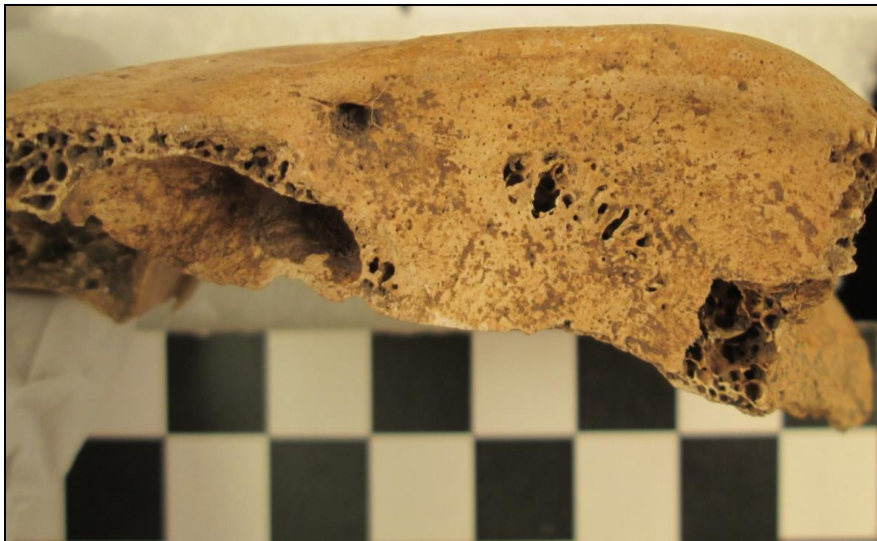
I sterilized all surfaces, sampling instruments, and tools with 91% isopropyl alcohol between samples. For each sample, I wore new gloves and used fresh pieces of aluminum foil, one large 2' x 2' piece to line the workspace on which the bone lay, and another 4" x 4" piece for sample collection. I extracted samples of cancellous bone just below the head and neck of humeri and femora by scraping the most proximal section of the medullary cavity using a metal spatula and pick. In some long bones the medullary cavity was accessible through fragmented sections of proximal cortical bone. Other bones that were complete on the proximal end but fragmented on the distal end required long metal picks which were used to scrape deep in the cavity to collect bone samples. I recovered 0.25-0.5g of bone per element sampled. I sealed samples tightly in small foil packets and placed them in appropriately labeled sealed plastic containers.

***Photography.***

I photographed each bone prior to sampling. Where cranial fragments were present for the individuals selected for sampling, I photographed the cranial fragments to look for the presence of cribra orbitalia (CO) and porotic hyperostosis (PH) (See Figure 4.8.1 A, B). I noted the presence and state of these lesions where available (see Table 4.1). Where I observed hemozoin-like material during the sampling process, I placed the material on a filter paper and examined it under the microscope. I

photographed the sample in high magnification (50X -500X) to further document the nature of the hemozoin-like material (See Figure 4.8.2 A.B).

Figure 4.8.1 (A,B) Cribra Orbitalia and Porotic Hyperostosis in YPM PA 173 Burial 1

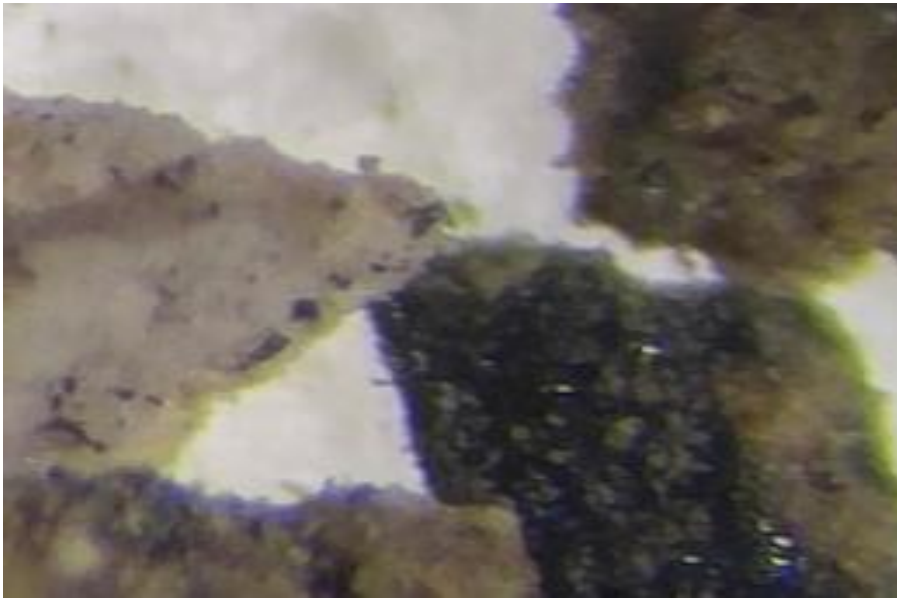


(A-above) Active Cribra Orbitalia

(B-below) Porotic Hyperostosis in YPM PA173 B1



Figure 4.8.2 (A,B) Hemozoin-like Material and Trabecular Bone YPM PA151 B1, II



(A-above) Taken by author at 500x magnification (UofL Archaeology Laboratory)

(B-below) Taken by author at 400x magnification



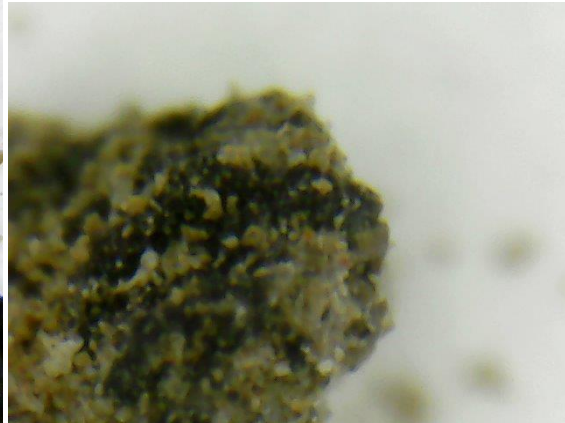
Figure 4.8.3 YPM PA152 B2, I2



Trabecular bone (brown) and Hz (black)  
(350x magnification)

Photo taken by Author

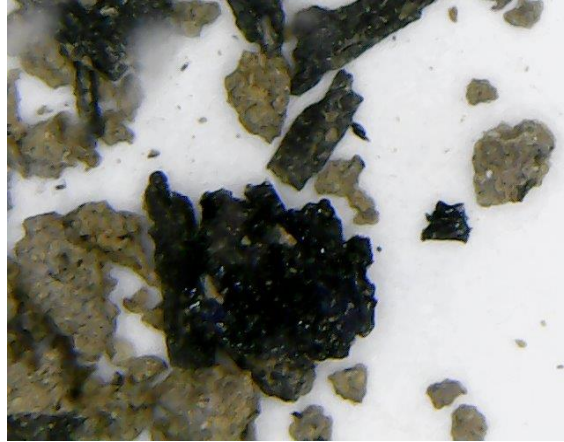
Figure 4.8.4 (A) YPM PA151 B1, I1



Trabecular bone (brown) and Hz (black)  
(400x magnification)

Photo taken by Author

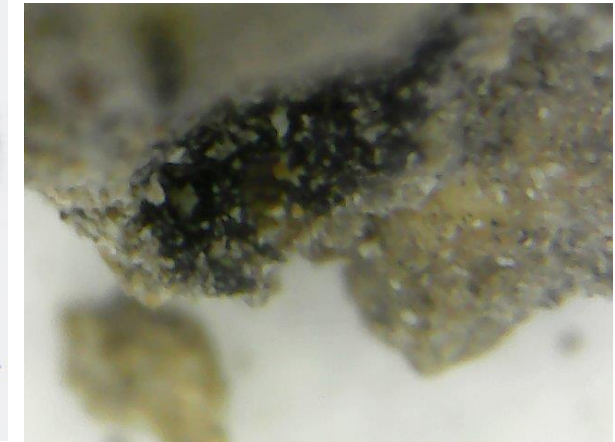
Figure 4.8.4 (B) YPM PA151 B1, I1



Trabecular bone (brown) and Hz (black)  
(300x magnification)

Photo taken by Author

Figure 4.8.5 YPM PA173 B1



Trabecular bone (brown) and Hz (black)  
(500x magnification)

Photo taken by Author



Figure 4.8.6 YPM PA166



Trabecular bone with Hz (black granules uniformly distributed on the surface of the bone) (400x magnification)

Photo taken by Author

*Methods of Identification.*

To look for molecular evidence of hemozoin, I first utilized mass spectrometry. This technique was employed specifically because it has been successful in identifying hemozoin in ancient skeletal remains (Inwood, 2017). However, a potential contamination issue arose using the mass spectrometer, therefore I selected infrared spectroscopy as an additional method of analysis. This technique is used in biomedical contexts for identifying hemozoin and was adopted for the purposes of this research. The final analysis performed was scanning electron microscopy to magnify the sample in order to characterize and measure individual hemozoin crystals. Unique challenges were presented with each analysis. The steps involved in each analysis, as well as the caveats and results are presented in the remaining chapters of this thesis.

### *MALDI tof MS.*

Inwood (2017) determined Matrix-Assisted Laser Desorption/Ionization time-of-flight Mass Spectrometry (MALDI tof MS) to be the most effective instrument when detecting *Plasmodium*-produced hemozoin crystals in skeletal remains. The MALDI tof MS operates by firing a 337nm nitrogen laser at the sample to charge the particles (i.e. ionization) and a detector measures the components to generate a mass spectrum. These 'spectra' are used like fingerprints in identification. The data appear as peaks on an X-Y plane, referred to as a mass spectrum, and units of measurements are (m/z), read as 'mass-to-charge ratios' (Wilkins and Lay 2005). A complete identification profile is present for a molecule when the 'molecular ion' is identified in the spectra. A partial identification profile is present when the 'fragmentary ions' are identified. The molecular ion for hemozoin is 615/616 (m/z), and associated fragmentary ions for hemozoin include 498, 512, 526, 556, 571(m/z) (Demirev, 2002). The intensity of the peaks should be over 30% to say with certainty the hemozoin is parasite produced and blood-born, rather than preserved heme (Laposki, 2017).

### *Sample Preparation for MALDI tof MS.*

Samples were prepared in the basement of 51 Hillhouse in the Yale University Archaeology Laboratory (YUAL) according to the protocol used by Inwood (2017). An additional step was added to the protocol utilized by Inwood (2017) prior to grinding down the sample in a mortar and pestle. This step included placing the sample on a filter paper, into a petri dish, and under (300-450x) magnification using a digital microscope (Colemeter AE 7900). I used a pair of tweezers and a scalpel to remove any visible bone from pigmented material, to increase the yield and improve the results. This step is not

included in Inwood (2017) protocol, and is therefore an original method currently being refined in University of Louisville's Archaeology Laboratory.

I weighed 250mg of cancellous bone on sample paper and then ground it into a fine powder using a mortar and pestle. First, 5mg of ground bone powder was measured in an Eppendorf tube, and I used a pipette to add 5mL of hydrochloric acid (HCL) to the tube. Then, the Eppendorf tube was vortexed until the bone appeared suspended in the HCL, which took about three minutes on average. The resulting solution was dropped onto the plate and run on the MALDI. This preparation protocol did not yield clear spectra, thus an amended protocol was used. In the 2nd protocol, I substituted the hydrochloric acid for 0.1% trifluoroacetic acid (TFA). Also, the samples were prepared in the same ratio (1:1) but in smaller quantities. Specifically, I weighed 1 mg of ground bone powder in an Eppendorf tube and used a pipette to add 1mL of TFA to the tube. Then, the Eppendorf tube was vortexed until the bone appeared suspended in the HCL, which took about three minutes on average. I used a pipette to drop the resulting solution onto a 100-well MALDI plate, dropping 10 spots from each sample onto the plate, leaving one row of sample wells empty between samples from different individuals to prevent mixing and eliminate error. I allowed the sample-spotted plate to dry for 20 minutes before loading it into the instrument. The latter protocol using TFA and smaller quantities of material proved optimal. This preparation protocol was employed thereafter. It was used to prepare sample 12, which presented spectra identifying the molecular ion for hemozoin.

### *MALDI tof MS Analysis.*

I analyzed 32 samples using the MALDI tof MS in Yale's Chemical and Biophysical Instrumentation Center (CBIC). A Voyager DE Pro MALDI tof MS (Yale CBIC, New Haven, CT) was set to an intensity of 1,200 with a time delay of 100 ns to begin. The intensity of the laser was adjusted until the mass spectra appeared clean with low baselines and minimal background noise. The ideal intensity for all samples on this instrument was 1,467. The linear mode provided the clearest results and all plates were run in this mode. I set the instrument to fire 300 shots per run, and analyzed each spot on the plate three times. If I observed peaks for fragmentary ions, but not the 615-616 m/z molecular ion, I ran the sample three additional times with different laser intensities. Increasing the laser intensity by 15% improved the results in some samples. Data were compiled and reviewed using software provided at Yale's CBIC.

### *FTIR Spectroscopy.*

Fourier Transform Infrared (FTIR) Spectroscopy, a type of non-destructive analysis that can be utilized to identify and quantify molecular compounds, was used to analyze prepared samples for wavelengths corresponding to hemozoin. FTIR Spectroscopy has been used in clinical and biomedical settings to identify hemozoin (Egan, Mavuso, & Ncokazi, 2001; Lvova et al., 2016; Oliveira et al., 2007; Samson et al., 2012; Tempera et al., 2015; Webster et al., 2009). An FTIR spectrometer operates by shining multispectral beams of infrared light onto a sample and a receiver detects remnant wavelengths of light. The instrument can then infer the wavelengths of light absorbed by the sample. Analysis involves measuring the infrared wavelengths absorbed a sample, which are reported as peaks or bands on an X-Y spectrum plot (Figure 4.4). The amount of light absorbed by a sample at each wavelength is unique to its molecular



components, thus producing a ‘fingerprint’ spectrum that is used to identify individual components of a sample. Although an FTIR spectrometer allows for quantification of components that is beyond the scope of this research. I selected a diamond Attenuated Total Reflectance (ATR) interface to use with the FTIR spectrometer because this method is ideal for the analysis of solids and the sample size required is minimal (20-25 mg) (Wilkins and Lay 2005).

*Reference Spectra to Identify Hemozoin Using FTIR Analysis.*

Tempera et al. (2015) reported FTIR spectra for hemozoin with bands at 1664 and 1209 cm. Lvova et al. (2016) identified hemozoin with bands at 1660 and 1207 cm which identify the carbon-oxygen bond of the carbonyl group by the ferric iron. The 1660 and 1207cm bond is responsible for the structure of hemozoin at the molecular level (Oliveira et al., 2007). Samson et al. (2012) identified prominent bands for hemozoin at 1661 and 1206 cm, while Egan et al. (2001) found bands at 1663 and 1210 cm for purified hemozoin. Webster et al. (2009) identified three bands assigned to hemozoin including 1664, 1209 and 1712 cm. These signature hemozoin bands were used as a reference to interpret the results of FTIR analyses of samples from indigenous Caribbean skeletal remains.

*Experiments in ATR-FTIR Sample Preparation Protocols.*

I experimented with different protocols to prepare samples for ATR-FTIR analysis. Hollund et al. (2013) report that excessive grinding down of bone samples has negative effects on the spectra produced. I ground a sample of bone (0.1g) using a mortar and pestle with a minimal amount of force, and analyzed the sample. I repeated the process using the material from the same sample, but instead I used excessive amounts of

force to pulverize the bone sample. I observed no differences in the results for each sample, and neither spectra contained wavelengths indicative of hemozoin.

Next, I used a metal scalpel and tweezers to section a piece of bone presenting pigmented granules on the surface. With those tools I modified the shape and size of the sample to fit the ATR-FTIR diamond aperture. I placed a thin slice of bone directly onto the aperture, applied the press, and ran the analysis. The results indicated wavelengths for bone, but not for hemozoin. I did not observe any improvements in the results acquired using either of the experimental protocols; therefore I used a standard procedure which involved grinding of bone samples in a mortar and pestle prior to placing them on the diamond interface of the ATR-FTIR.

#### ***ATR-FTIR Analyses.***

I used an Agilent 4300 Handheld FTIR (UofL Archaeology Laboratory, Louisville, Kentucky) to analyze 17 samples representing 14 individuals to look for infrared wavelengths indicative of hemozoin (see Table 4.1). I ran all samples twice to corroborate results, with additional runs performed when contradictory or uncertain wavelengths were observed. I compiled the data using MicroLab software.

#### ***Scanning Electron Microscopy.***

Scanning electron microscopy (SEM) is used to in a wide range of magnifications from 25x to over 1,000,000X to characterize a material and record features as small as 1nm. SEM also has the ability to determine elemental composition of particles being examined as a result of the x-rays produced when the electron beam of the microscope strikes a target. If coupled with an x-ray analyzer the emitted x-rays are sorted by their energy or wavelength values, related to specific elements (Kobilinsky, 2012).

SEM analysis was conducted at the University of Connecticut's ThermoFisher Scientific Center for Advanced Microscopy and Materials Analysis (CAMMA), with the assistance of Dr. Roger Ristau, to analyze the size and morphology of the crystals in the samples which presented signatures for hemozoin using the MALDI tof MS. I selected samples in which the molecular ion was present in the mass spectra. A Teneo Low Vacuum SEM was utilized to analyze 8 samples representing 7 individuals from the Caribbean collection (Table 4.1). A guideline for differentiating between hemozoin crystals from each *Plasmodium spp.* as well as Schistosomiasis and Chagas, two additional parasites that produce hemozoin and inhabit the region under study, is provided by Oliveira et al. (2009) and Noland et al. (2003) and used to interpret the results gathered using SEM to analyze Caribbean skeletal samples (see Table 4.3).

CHAPTER 5: RESULTS AND DISCUSSION

Table 5.1 Results for Analyses Conducted

#	YPM Catalog ID	Sex	Age	Element	Crania	PH	CO	SEM	FTIR	MALDI	FTIR Hz 1206-1210	FTIR Hz 1660-1664	FTIR Hz 1713-1716	FTIR RBC 2800-3000	FTIR Hematin 1625
1	PA152 B2,I2	M	Adult	Femur	-	NA	NA	X*	-	-	NA	NA	NA	NA	NA
2	PA152 B3,I4	M	Adult	Femur	-	NA	NA	-	-	-	NA	NA	NA	NA	NA
3	*PA163 B2,IB	M	12-14	Humerus	+	+	NA	X*	-	X	NA	NA	NA	NA	NA
4	PA163 B2,IB	M	12-14	Femur	+	+	NA	-	X	-	X*	X*	-	-	X*
5	*PA161B1,IA	F	>25	Femur	+	+	+	X*	X	-	-	-	-	-	-
6	PA161B1,IA	F	>25	Femur	+	+	+	-	-	X	NA	NA	NA	NA	NA
7	PA161 B1,IA	F	>25	Tibia	+	+	+	-	X	-	X*	X	-	-	X*
8	*PA164 B1	F	>45	Femur	-	NA	NA	-	-	X	NA	NA	NA	NA	NA
9	PA164 B1	F	>45	Humerus	-	NA	NA	-	X	-	X*	-	-	-	-
10	PA152 B3,I1	M	>35	Humerus	-	NA	NA	-	X	-	-	X*	-	-	X-
11	*PA150 B1,I1	M	Adult	Humerus	+	+	NA	-	X	X	-	X*	X-	-	X-
12	*PA151 B1,I1	F	>35	Femur	-	NA	NA	X	X	X*	-	X*	X*	-	X-
13	PA151 B1,I1	F	>35	Tibia	-	NA	NA	X*	X	-	-	-	X-	-	-
14	*PA173 B1	M	Adult	Tibia	+	+	+	-	X*	-	X*	X*	X*	-	X*
15	PA173 B1	M	Adult	Femur	+	+	+	-	X*	-	X*	X*	X*	-	X-
16	PA173 B1	M	Adult	Humerus	+	+	+	X*	X	X	-	X-	-	-	-
16.5	PA173 B1	M	Adult	Tibia	+	+	+	-	X	-	-	X*	-	-	-
17	PA170	M	Adult	Femur	-	NA	NA	-	X	X	X*	X*	X	-	X*
18	PA165 B1,I1	NA	Adult	Humerus	-	NA	NA	-	X	-	X*	X*	-	X	X*
19	*PA162 B2	NA	2.5 y.o.	Femur	-	NA	NA	X	-	-	NA	NA	NA	NA	NA

20	PA162 B2	NA	2.5 y.o.	Femur	-	NA	NA	-	X	-	X*	-	-	-	-
21	PA150 B1,I1	M	Adult	Humerus	-	NA	NA	-	-	X	NA	NA	NA	NA	NA
22	PA153 B2,I4	NA	5 y.o.	Humerus	-	NA	NA	-	X	-	-	X-	-	-	-
23	PA153 B2,I2	M	Adult	Ulna	-	NA	NA	-	-	X	NA	NA	NA	NA	NA
24	PA153 B2,I3	NA	1 y.o.	Radius	-	NA	NA	-	-	X	NA	NA	NA	NA	NA
25	PA163 B2,IA	NA	Adult	Ulna	-	NA	NA	-	-	-	NA	NA	NA	NA	NA
26	PA163 B2,IB	M	12-14	Ulna	-	NA	NA	-	-	-	NA	NA	NA	NA	NA
27	PA165	NA	Child	Radius	-	NA	NA	-	-	-	NA	NA	NA	NA	NA
28	PA165	NA	6 y.o.	Ulna	-	NA	NA	-	X	-	X*	-	-	-	X*
29	PA166	NA	Child	Radius	+	+	NA	X	-	-	NA	NA	NA	NA	NA
30	PA166	M	>50	Femur	-	NA	NA	-	-	X	NA	NA	NA	NA	NA
31	PA167	M	Adult	Ulna	-	NA	NA	-	-	-	NA	NA	NA	NA	NA
32	PA161 B1,IA	F	>25	Femur	+	+	+	-	-	-	NA	NA	NA	NA	NA

Key: YPMNH –Yale Peabody Museum of Natural History

PO-Porotic hyperostosis

CO-Cribra Orbitalia

(+)- Presence of lesion or element

(-)- Absence of lesion or element

(\*YPM Catalog ID) -more than one sample taken from individual

RBC- Red blood cell

(X-)- Peak present but slight

(X)- Peak present and clear

(X\*)- Peak present as a shoulder

y.o.- Years old

Hz- Hemozoin

WL- Wavelength

B- Burial (A,B,1,2,..)

I-Individual

Ind-Indeterminate

NA-Not available

SEM- Scanning Electron Microscope

FTIR- Fourier Transform Infrared Spectroscopy

MALDI- Matrix Assisted Laser Desorption/Ionization

time-of-flight Mass Spectrometry

F-Female

(X)- Analysis conducted

(X\*)- Results identifying hemozoin

M-Male

PO-Porotic hyperostosis

Element- Bone sampled

Sources: Tempera et al. (2015), Lvova et al (2016),Oliveira et al. (2007), Samson et al. (2012) Egan et al. (2001), Webster et al. (2009)

## ***Results***

### ***MALDI tof MS Analyses.***

I analyzed 32 samples using a MALDI tof MS in Yale's Chemical and Biophysical Instrumentation Center. Demirev (2002) established the identification criteria for hemozoin using a MALDI tof MS as the molecular ion (615 m/z) and fragmentary ions (484, 498, 512, 526, 557, 571 m/z). Using these criteria, I was able to identify the molecular ion and all of the fragmentary ions for hemozoin in sample 12, a female adult (PA 151 Burial 1, Individual1) recovered at the site of Toa Baja below Santa Elena style ceramics, which were radiocarbon dated to AD 890-AD 1210 (Rouse and Allaire, 1978). Results from these analyses showed molecular ions and fragmentary ions for hemozoin in a majority of the samples analyzed; however, due to a possible contamination issue after analysis of the first sample, data for the remaining 31 are considered inconclusive. Adaptation of the sample preparation protocol for identification of hemozoin in samples of cancellous bone tissue with a MALDI tof MS should improve results in the future, but such refinements are beyond the scope of the current project. Currently, I am accepting the results from sample 12 because it was prepared and analyzed first. Our current understanding is that this sample may have contaminated the remainder of the samples, which results have been discarded for this reason. To further investigate these results, additional testing of this set of samples was conducted with ATR-FTIR and SEM, which have also been shown to be effective in the detection of hemozoin (Egan et al. 2001, Lvova et al 2016, Noland et al. 2003, Oliveira et al. 2007, Oliveira et al. 2009, Tempera et al. 2015, Samson et al. 2012, Webster et al. 2009).

Figure 5.1 MALDI tof MS Results (Sample 12) YPM PA 151B1, I1

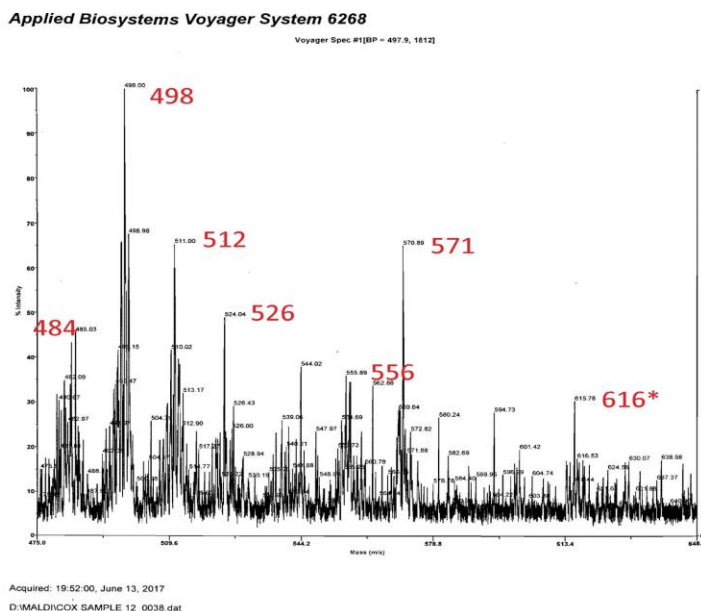


Figure 5.1: Y axis= intensity, X axis= (m/z) mass-to-charge-ratios. The 616\*peak indicates the presence of the molecular ion for hemozoin; 484,498,512,526,556, and 571 are fragmentary ions associated with hemozoin.

### ***Results of ATR-FTIR Analyses.***

I analyzed 17 samples using ATR-FTIR in University of Louisville's Archaeology Laboratory (see Table 4.1). The spectra acquired presented 'shoulders' (see Figure 5.2(A)) around the expected wavelengths for hemozoin. This is a known problem with FTIR when multiple components are present that produce overlapping wavelengths. This suggests that carbon and phosphate are masking potential hemozoin wavelengths. The dominant wavelengths for bone overlap with those of hemozoin, which makes sense in this research because these samples were extracted from archaeological bone.

In addition, I observed wavelengths indicative of bone marrow and red blood cell lipids. These results are interesting due to the pathophysiology of malaria in the human host. Hemozoin is formed within the red blood cells; hence, finding these remnants of red

blood cells in association with pigmented granules in skeletal remains is in accordance with clinical knowledge of disease progression. Although these components are involved in other disease processes, they are not in conflict with the processes involved in hemozoin production.

Shoulders for hemozoin, rather than distinct peaks, are present in 8 of the samples analyzed using ATR-FTIR. Both the 1209 and 1660  $\text{cm}^{-1}$  peaks should be present to identify hemozoin. For sample #7 (YPM-PA161 B1, IA), the 1660  $\text{cm}^{-1}$  peak is present but not the 1209 $\text{cm}^{-1}$ . These results are not conclusive for hemozoin without both peaks for the iron carboxylate bond (1209  $\text{cm}^{-1}$  and 1660  $\text{cm}^{-1}$ ). However, they do suggest a need to continue the investigation of this collection for evidence of hemozoin with additional methods of analysis. With respect to the suitability of ATR-FTIR to the goals of this project, the results indicate a need for refinement of sample preparation protocols to eliminate bone minerals and other biological residues prior to further attempts at hemozoin identification in ancient skeletal remains.



Figure 5.2 (A) ATR-FTIR Results for YPM- PA170 (Sample 17)

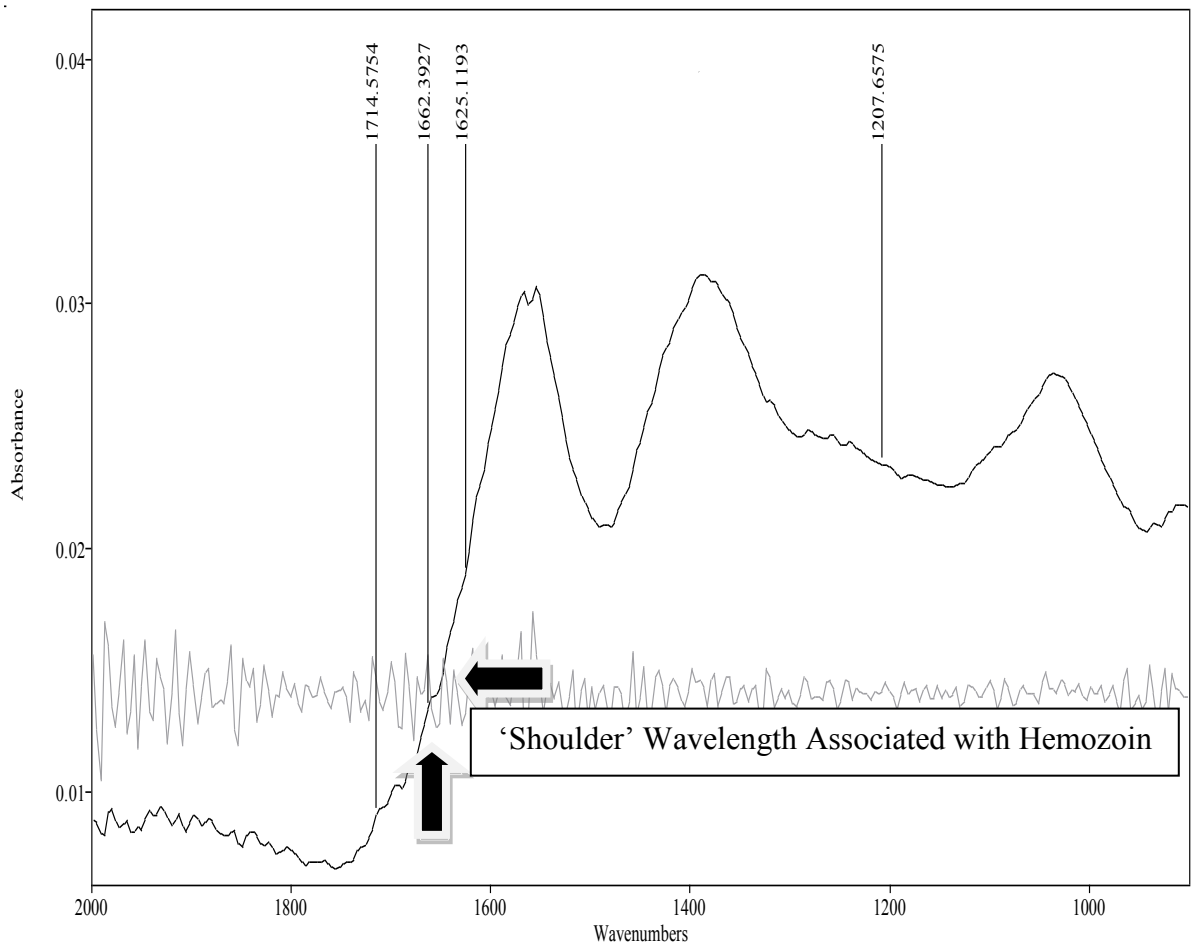


Figure 5.2(A): Mass spectrum demonstrating the presence of a 'shoulder' wavelength (black arrows) for hemozoin in YPM- PA170 (Sample 17).

Figure 5.2 (B) ATR-FTIR Results for YPM PA165 B1, I1 (Sample 18)

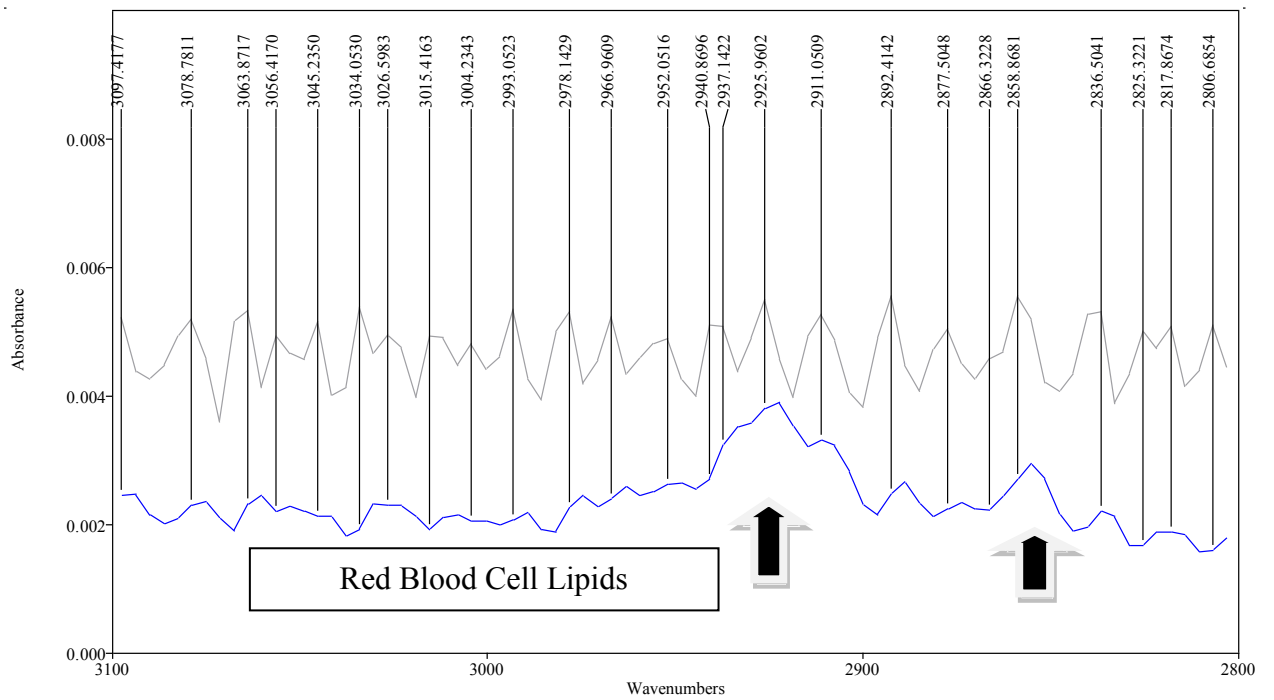


Figure 5.2B: Mass spectrum demonstrating wavelengths for red blood cell lipids in YPM PA165 B1, I1

As referenced in (Webster et al., 2009)

### ***Results of Scanning Electron Microscopy Analyses.***

Eight samples were analyzed using SEM: seven were imaged using a Teneo Low Vacuum SEM, and one sample was analyzed and imaged using a FEI Helios 460 SEM. Hemozoin crystals were identified in samples 1,3,5,12,13, and 16. From amongst these, all but samples 3 and 5 presented rectilinear hemozoin crystals indicative of *Plasmodium spp.* malaria. Samples 3 and 5 demonstrate hemozoin crystals, but they are circular, thus the causative parasite was most likely *Schistosoma spp.* or *Trypanosoma cruzi* (Fig 5.6 and 5.7 (B)). In addition to hemozoin crystals, SEM analyses revealed what might be parasite bodies for *Schistosoma mansoni* and *Trypanosoma cruzi*. Specifically, in sample 5 there is an identifiable flagellum from *Trypanosoma spp.* (Figure 5.7 (A)) and in sample 19 I observed what might be the oral and ventral suckers for a *Schistosoma mansoni* parasite (Fig 5.9.2). Preliminary observations are detailed below.

Table 5.1 Results of Scanning Electron Microscopy

Sample #	Figure #	Presence of Hemozoin	Hemozoin Crystal Dimensions	Causative Species of HZ Crystals
1	4.8.3 5.3 5.4 5.5	X	200nm, 500nm, 400nm	<i>P. Malariae</i> * <i>Plasmodium spp.</i>
3	5.6	X	50-1000nm	<i>Schistosoma spp.</i>
5	5.7.1 5.7.2	X	50-1500nm	<i>Schistosoma spp. Or</i> <i>Trypanosoma spp.</i>
12	4.8.4 4.8.5 5.7.2	X	100nm	<i>P. vivax</i> *
13	5.8.2	X	400-500nm	<i>Plasmodium spp.</i>
16	4.8.6 5.8.3	X	500nm	<i>Plasmodium spp.</i>
19	4.8.7 5.8.4	—	—	—
29	5.8.5	—	—	—

\* indicates species estimation has been made based upon crystal dimensions (Noland et al. 2003; Oliveira et al. 2005).

## **Discussion**

### ***Caveats of FTIR Analysis: the Bone and Zoin' Problem.***

Previous and contemporary research to identify and characterize hemozoin using ATR-FTIR is conducted in clinical and biomedical settings wherein samples are isolated from blood, bone marrow, livers, the guts of mosquitoes, and other contexts. This is not the case for archaeological bone. One complication of using FTIR- ATR to identify hemozoin in archaeological bone samples is that bone generates wavelengths in the infrared spectrum that overlap with those wavelengths identifying hemozoin including the amide at 1640, carbon at 1415 and phosphate at 1215(Hollund, Ariese, Fernandes, Jans, & Kars, 2013). The two different set of wavelengths are so close on the spectra that one 'wave' may be hidden behind another (see Figure 4.6 for wavelengths associated with bone). My samples are archaeological and have been extracted from the medullary cavities of bones thousands of years old. Many of the samples were recovered from exposed cavities and contain additional sediments, and therefore require additional processing to separate potential hemozoin from tissue, sediments, and other residues.

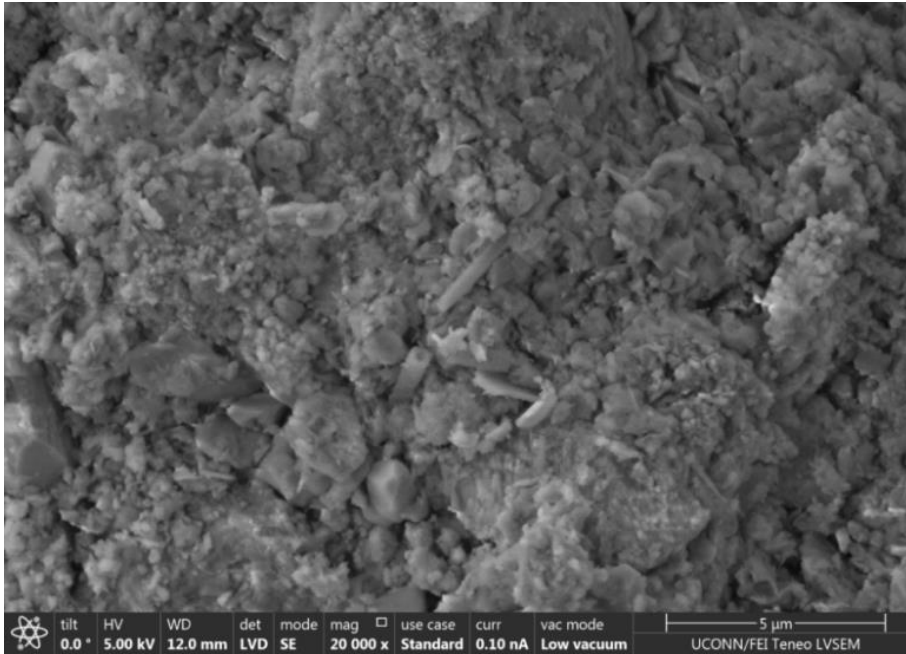
### ***Observations Associated with MALDI tof MS and Hemozoin.***

In accordance with the results of recent work by Inwood (2017), the results of this project suggest mass spectrometry as an effective way to identify hemozoin in archaeological bone samples. However, issues of contamination may arise, the etiology of which has yet to be identified. The only difference in my altered sample preparation protocol is the absence of a matrix, prior to dropping the samples on the plate. This may or may not be related to the issues of contamination, but like other caveats that have surfaced throughout this project, will be explored further in the future research.

*Caveats of MALDI tof MS Analysis of Archaeological Samples.*

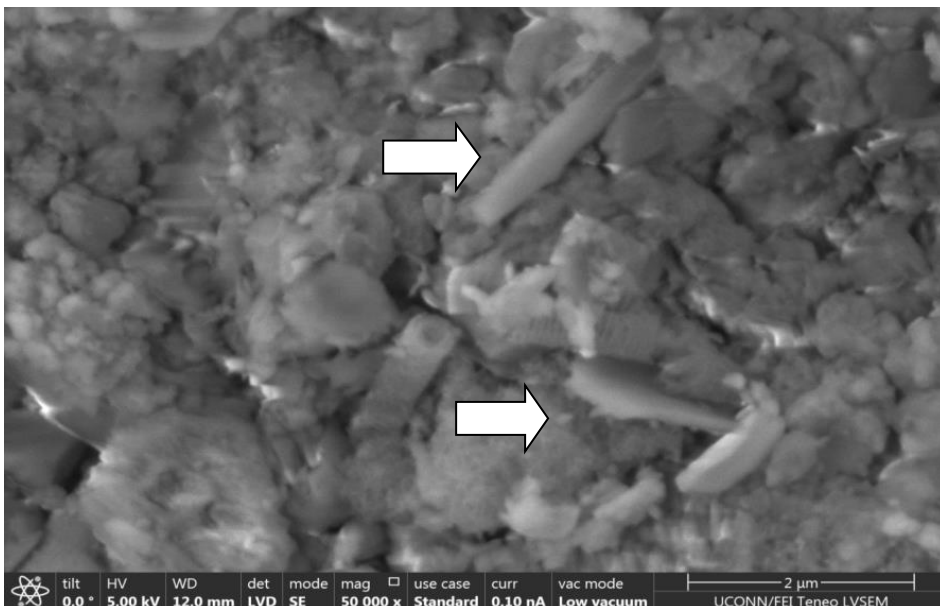
In accordance with the results of recent work by Inwood (2017), the results of this project suggest mass spectrometry as an effective way to identify hemozoin in archaeological bone samples. However, issues of contamination may arise, the etiology of which has yet to be identified. The only difference in my altered sample preparation protocol is the absence of a matrix, prior to dropping the samples on the plate. This may or may not be related to the issues of contamination, but like other caveats that have surfaced throughout this project, will be explored in future research.

Figure 5.3 YPM PA152 B2, I2 (Sample 1)



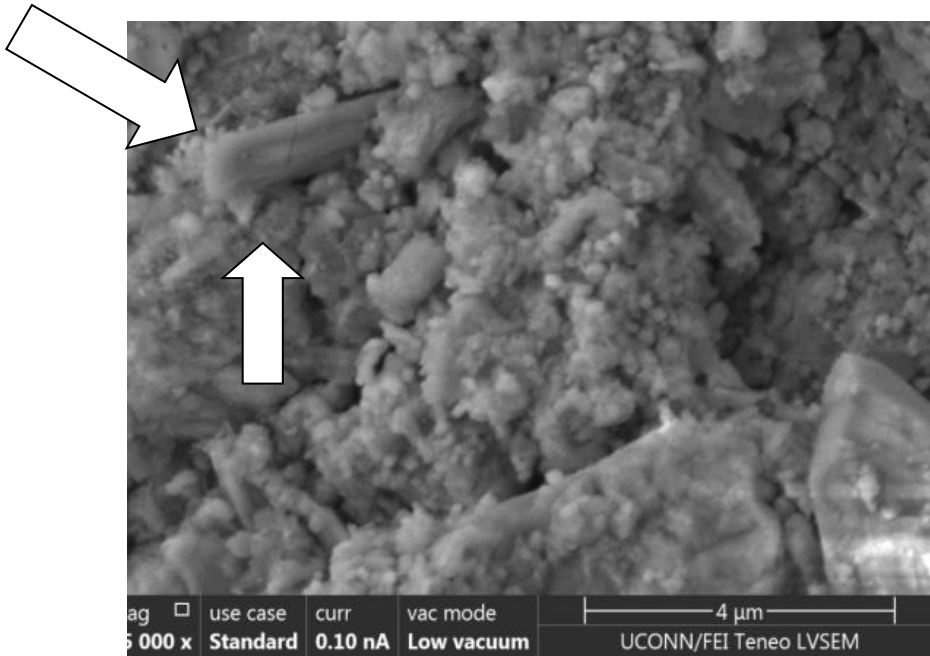
Scanning electron microscope image of sample from YPM PA152 B2, I2  
*Plasmodium spp.* Hz crystals (20,000x magnification)

Figure 5.4 SEM YPM- PA152 B2, I2 (Sample 1)



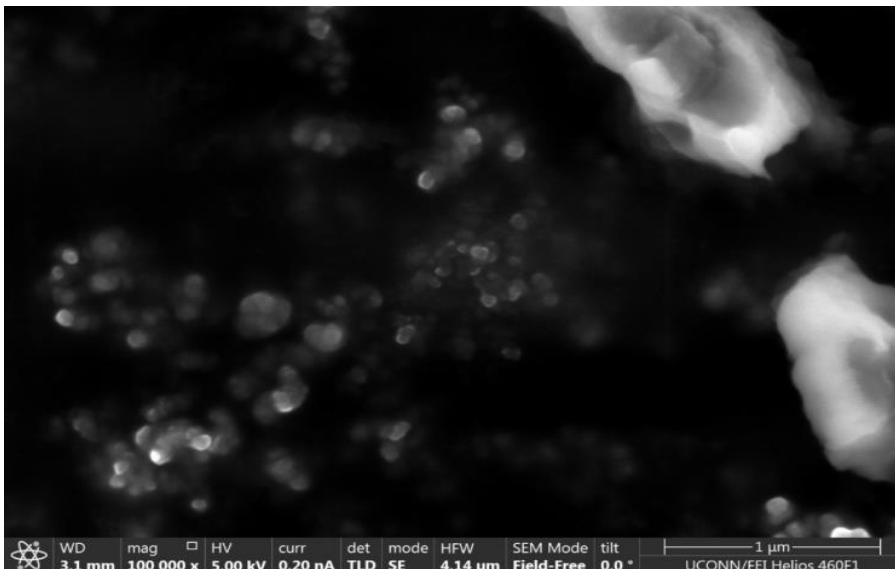
Scanning electron microscope image of sample from YPM PA152 B2, I2  
*Plasmodium spp.* Hz crystals 200nm (50,000x magnification)

Figure 5.5 SEM YPM PA152 B2, I2 (Sample 1)



Scanning electron microscope image of sample from YPM PA152 B2, I2  
*Plasmodium spp.* Hz crystals 500nm (60,000x magnification)

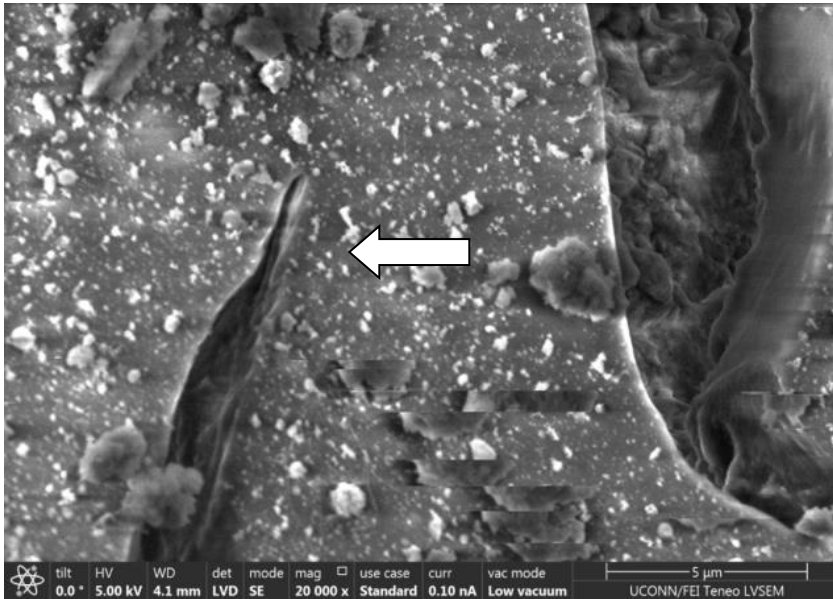
Figure 5.6 SEM YPM PA163 B2, IB (Sample 3)



Scanning electron microscope image of sample from YPM PA163 B2, IB  
*Schistosoma mansoni* or *Trypanosoma cruzi* Hz crystals; crystals range from 50-1,000nm  
in diameter (100,000x magnification)



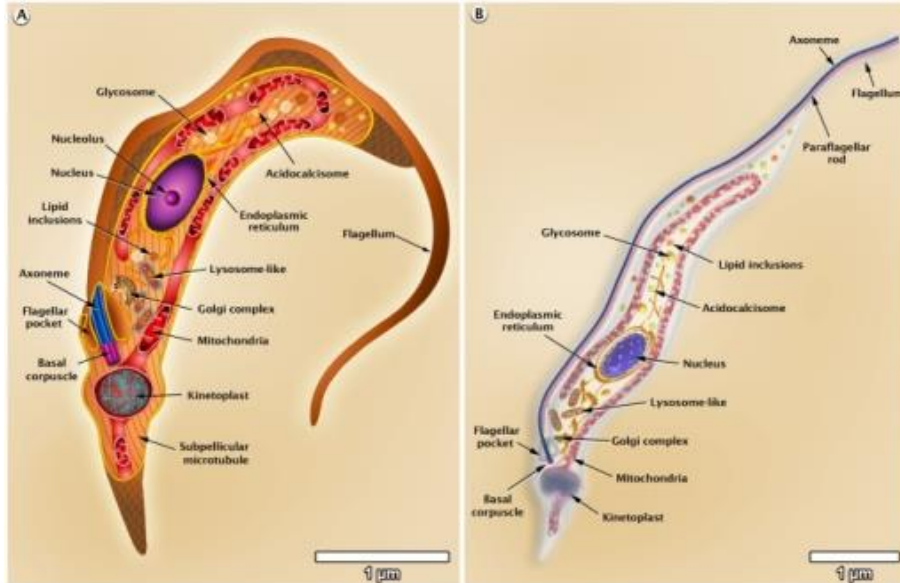
Figure 5.7 (A) SEM YPM PA161 B1, IA (Sample 5)



Scanning electron microscope image of sample from YPM PA161 B1, IA  
(20,000x magnification)

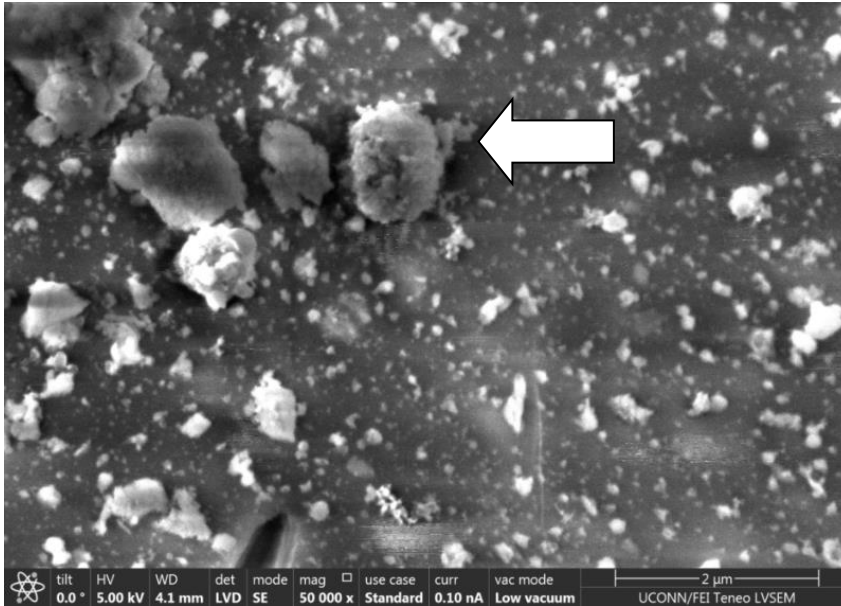
Possible flagellum from a *Trypanosoma cruzi* parasite body (see below)

*Trypanosoma cruzi*, (the causative agent of Chagas disease) (Reference)



Source: Teixeira, Benchimol, Crepaldi, & de Souza, 2012

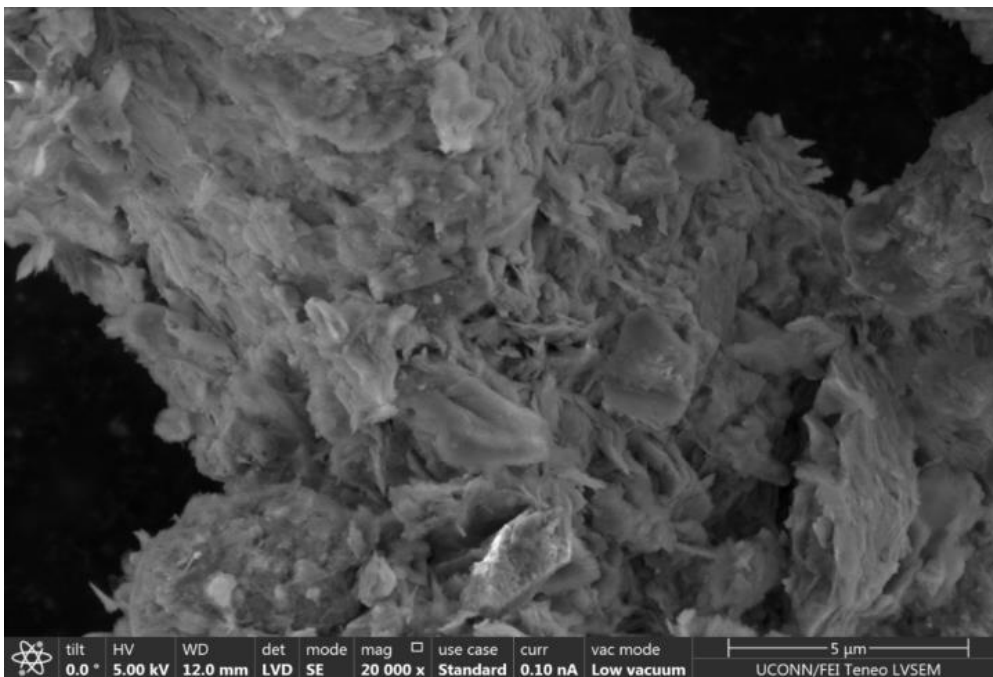
Figure 5.7 (B) SEM YPM PA161 B1, I1 (Sample 5)



Scanning electron microscope image of sample from YPM PA161 B1, I1

*Schistosoma spp.* or *Trypanosoma spp.* Hz crystals; crystals range from 50-1,500nm in diameter

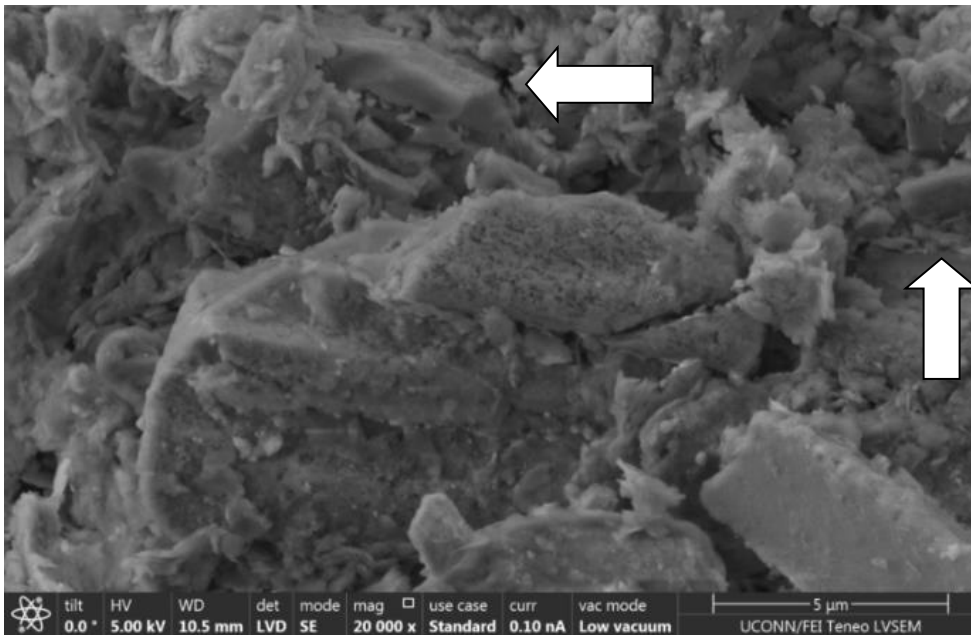
Figure 5.8 SEM YPM PA151 B1, I1 (Sample 12)



Scanning electron microscope image of sample from YPM PA151 B1, I1

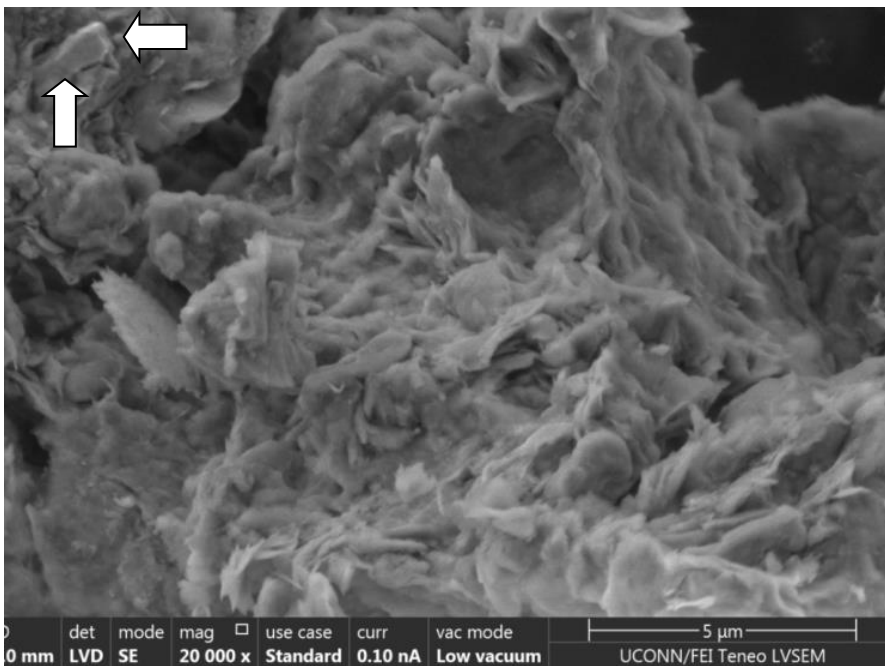
*Plasmodium spp.* Hz crystals (20,000 x magnification)

Figure 5.9 SEM YPM PA151 B1, I1 (Sample 13)



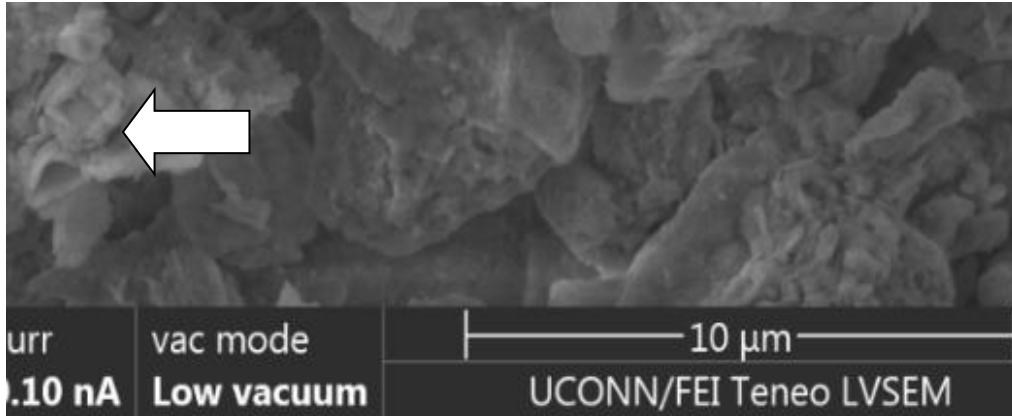
Scanning electron microscope image of sample from YPM PA151 B1, I1  
*Plasmodium spp.* Hz crystal 400-500nm - (20,000x magnification)

Figure 5.9.1 SEM YPM PA173 B1 (Sample 16)



Scanning electron microscope image of sample from YPM PA173 B1  
*Plasmodium spp.* Hz crystal 500nm - (20,000x magnification)

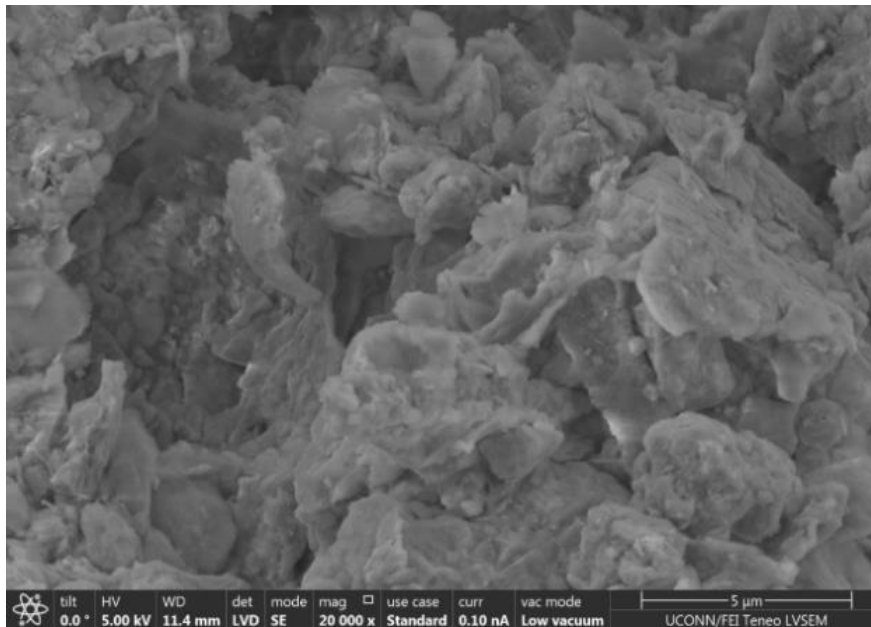
Figure 5.9.2 SEM YPM PA162 B2 (Sample 19)



Scanning electron microscope image of sample from YPM PA162 B2

Possible oral and ventral suckers of *Schistosoma mansoni* parasite 1000nm (wide)  
(20,000x magnification)

Figure 5.9.3 SEM YPM PA1 (Sample 29)



Scanning electron microscope image of sample from YPM PA166

No observable Hz crystals- (20,000x magnification)

## CHAPTER 6: CONCLUSION

The Caribbean collection of skeletal remains at Yale's Peabody Museum of Natural History provides a rich opportunity to contribute bioarchaeological and paleoepidemiological data towards the broader understanding of the lifeways and health of indigenous Caribbean populations. The results of this investigation suggest that *Plasmodium spp.* were present in the Americas and infecting Caribbean populations as early as AD 1000. However, the initial introduction of malaria to the Americas is likely to have been much earlier, as suggested by the *Plasmodium spp.* antibodies isolated in South American mummies nearly 5000 years old (Gerszten et al., 2012). Based upon all available data, I postulate *P. vivax* and *P. malariae*, as well as *Schistosoma mansoni* and *Trypanosoma cruzi* to be the causative species for the hemozoin crystals observed in Yale's Caribbean Collection. Furthermore, if contemporary epidemiological trends are indicative of trends occurring in the past, then it would not be surprising if *P. simium* or *P. brasilianum* hemozoin crystals are eventually identified in the samples from Puerto Rico. To my knowledge there are no current reference samples of non-human primate malaria species hemozoin crystals isolated from human hosts, but once available, these data will greatly improve the aspects of accurately identifying hemozoin at the species level using scanning electron microscopy.

This research has shown that hemozoin detection is a promising method for the identification of malaria in other ancient populations where the disease is hypothesized

and aDNA is either inaccessible or poorly preserved. These results suggest a number of future directions for understanding the epidemiological curve of *Plasmodium spp.* in the Americas. A follow-up study with increased sample size and the geographical and temporal range represented would further substantiate these data. Ideally, skeletal remains that include cranial fragments recovered from multiple islands would be included. Isotopic analysis to discern possible dietary deficiencies would contribute toward an understanding of population health and skeletal manifestations thereof. Additionally, studies of samples of multiple sections of various bones would be worth exploring, given that our understanding of that pathophysiology of the disease suggests hemozoin could be deposited in any cavity with bone marrow. With larger sample sizes, mass spectrometry, a rapid method, would be used to identify samples presenting the molecular ion for hemozoin; these would then be analyzed using scanning electron microscopy coupled with aDNA to identify the parasite. The results would be then compared with the anemia-related skeletal lesions observed in the crania, thus contributing to the understanding of these lesions in ancient remains.

This study detecting *Plasmodium spp.* hemozoin in Caribbean skeletal remains changes our understanding of the global spread of malaria. This research contributes towards the development of a more complete epidemiological curve for *Plasmodium spp.*, which should include the Caribbean Islands before the arrival of Europeans. Combined with phylogenetic data and immunohistological evidence for *Plasmodium* antibodies, these results suggest the need to amend contemporary paradigms of the introduction of malaria to the Americas. In particular, if data emerges that supports the presence of malaria in the Andes and the Caribbean, 5000 and 1000 years ago respectively, other

routes of introduction should be considered including early waves of colonizers from the Eurasian landmass.

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Russian Language and Culture summer-immersion program ..... 06/2014-08/2014  
**St. Petersburg State University**, St. Petersburg, Russia  
David L. Boren Scholarship to Russia for an academic year ..... 09/2012-04/2013  
**California State University Northridge**, Northridge, CA  
STARTALK-  
Russian Language and Culture summer-immersion program ..... 06/2012-08/2012  
**Motlow State Community College**, Cookeville, TN ..... 09/2006-12/2006  
**Tennessee Technical College**, Lynchburg, TN  
General Educational Development Diploma ..... 05/2004

### **FIELD EXPERIENCE**

07/2015-09/2015 Archaeological field school, Middle Paleolithic cave site ‘Lapo do Picareiro’ in Portugal. Acquired training and experience in excavation, identifying stratigraphic layers, screening of sediments, cleaning and curation of artifacts recovered; recording and photography of features. Received training and experience operating a total station.

### **HONORS AND ACHIEVEMENTS**

Mary K. Tachau Essay Contest, Graduate winner, University of Louisville-(\$500) 2017  
David Ernst Fellowship, Smithsonian Tropical Research Institute- (\$4,400) .....2017  
1<sup>st</sup> place Graduate Oral Presentation,  
Kentucky Academy of Sciences, annual meeting .....2017  
Research Award, Anthropology Department, University of Louisville- (\$1,500) .....2017  
University of Louisville Research & Creative Activity Grant- (\$500) .....2017  
Travel Award, Anthropology Department, University of Louisville- (\$939) .....2016  
CIEE Language Intensive Focus Track (LIFT) Scholarship, Russian – (\$5,000) .....2013

David L. Boren Scholarship to Russia- (\$20,000) .....	2012
Overseers Honors Development Award- (\$1,600) .....	2012
University of Louisville Modern Language Fund (MLF) Award – (\$700) .....	2012

**LANGUAGES**

English: Native Language

Russian: Advanced Listener, Intermediate Speaker, Intermediate Reading & Writing

Spanish: Novice Listener and Speaker

**PROFESSIONAL ORGANIZATIONS**

American Association of Physical Anthropologists .....	2016-P
Boren Forum .....	2013-P
Golden Key Honor Society.....	2012-P
Kentucky Academy of Science .....	2011-P
Lambda Alpha.....	2013-P
National Society of Collegiate Scholars .....	2012-P
Paleopathology Association.....	2016-P
Sigma Alpha Lambda .....	2012-P
Sister Cities of Louisville.....	2012-P

**STUDENT ORGANIZATIONS- UNIVERSITY OF LOUISVILLE**

Anthropology Graduate Student Assoc.- (President) 2017- P, (Vice-President) 2016-2017	
Representative for the Anthropology department- Graduate Student Council .....	2016-2017
Advocacy and Involvement Committee- Graduate Student Council.....	2016-2017
Grant Writing Academy- School of Interdisciplinary and Graduate Studies .....	2017
Russian Language and Culture Club- (President) .....	2012-2013

**COMMUNITY SERVICE (VOLUNTEER)**

Angel Tree Initiative, UofL (AGSA),	
Co-sponsor-UofL Women’s Center- fundraiser .....	2017
Skeletal Forensics Workshop, K-12, Kentucky Science Center.....	2017
Kentucky Academy of Sciences, annual meeting, registration booth assistant.....	2017
STEM Girls in Science summer camp, presenter and assistant.....	2017
The Hermitage Museum, St. Petersburg, Russia - Archaeology Department .....	2012-2013
"English Tea" Language Learning Club, St. Petersburg, Russia – translator.....	2012
Disability Resource Center- University of Louisville - note-taker .....	2011
Salvation Army, Louisville, KY - soup kitchen .....	2010-2011

**PROFESSIONAL DEVELOPMENT**

Poster presenter, Malaria Symposium,	
American Assoc. of Physical Anthropologists .....	2018
Poster presenter, Kentucky Academy of Sciences, annual conference .....	2017
3 Minute Thesis competition participant, University of Louisville.....	2017
American Association of Physical Anthropologists- conference attendee.....	2016, 2017
Paleopathology Association- conference attendee.....	2016, 2017
Kentucky Heritage Council, 34th Annual Archaeological Conference.....	2017

Paleopathology Workshop, University of North Carolina,  
Chapel Hill-participant.....2015

**EMPLOYMENT**

Graduate Research Assistant, University of Louisville ..... 2017-P  
Burger Boy Diner, Louisville, KY, Cook (full-time/part-time) ..... 05/2013- 06/2015  
Third Avenue Café, Louisville, KY, Cook (full-time) ..... 02/2010- 11/2011  
Caregiver for an elderly woman, Smyrna, TN (full-time) ..... 12/2008- 01/2013

**REFERENCES**

Available upon request