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What Remains: Genetic and Epigenetic Correlates of Sociopolitical Change and the Ulterior Traces of Power.

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What Remains: Genetic and Epigenetic Correlates of Sociopolitical Change and the Ulterior Traces of Power.

by

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Dedication

For my kin.

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What Remains: Genetic and Epigenetic Correlates of Sociopolitical Change and the Ulterior Traces of Power.

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Recent developments in queer and feminist materialisms have offered productive ways to rethink the connections between nature and culture, and how these forces are mutually entailed in the constitution of bodies. These insights hold radical potentials for reconfiguring what science can mean and for remaking the worlds it helps to materialize. However, such perspectives are rarely taken as entry points for the production of scientific knowledge. Drawing upon emerging scholarship from queer, feminist, and indigenous theorists, this dissertation aims to take on the genetics lab as a site of political transformation. Here, I develop and apply new approaches for recovering the genetic and epigenetic correlates of sociopolitical change, showing that bodies are a "shifting entanglement of relations" (Barad 2007) between sociopolitical and material forces.

I begin by evaluating the boundary-making practices and conditions of possibility through which the field of population genetics has materialized certain indigenous bodies and histories to the exclusion of others. This research demonstrates how conventional population genetic research in North America, long predicated on notions of "biological purity", has helped to maintain the sociopolitical conditions of the settler state. Working from tribal and First Nations self-definitions, this research brings attention to histories that have been hidden in previous population genetic studies in the Americas. This work further

destabilizes notions of “indigenous DNA” as the sole criteria for indigenous belonging, through which settler claims to indigenous bodies and cultural heritage have unfolded in recent decades (TallBear 2013).

Next, I developed and evaluated methods for reconstructing chemical modifications to DNA, known as cytosine methylation, in five ancient genomes. Because changes in methylation can be shaped by social and environmental factors, reconstructing cytosine methylation in DNA from ancient people could help recover aspects of their lived experiences, shedding new light on past lifeways. I applied paleoepigenetic approaches to evaluate archaeologically-informed questions about the Wari society, the first expansive state in the central Peruvian Andes. By reconstructing ancient methylation patterns from 14 individuals who lived before and after the decline of the Wari state, I show that changes in DNA methylation trace sociopolitical and environmental changes in the ancient world.

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INTRODUCTION

> "What approaches might enable us to hold the politics of science and the possibilities of biology in the same frame, such that our new conceptions of materiality reflect the breadth of feminist and other contributions to knowing bodies?"
>
> **-Angela Willey, "Undoing Monogamy" (2016)**

> "Matter does not refer to a fixed substance; rather, matter is substance in its intra-active becoming – not a thing but a *doing*, a congealing of agency."
>
> **-Karen Barad, "Meeting the Universe Halfway" (2007)**

> "Nature is not a physical place to which one can go, nor a treasure to fence in or bank, nor an essence to be saved or violated. Nature is not hidden and so does not need to be unveiled. Nature is not a text to be read in the codes of mathematics and biomedicine. It is not the "other" who offers origin, replenishment, and service. Neither mother, nurse, nor slave, nature is not a matrix, resource, or tool for the reproduction of man… Nature is a topic of public discourse on which much turns, even the earth."
>
> **-Donna Haraway, "The Promises of Monsters" (1992)**

The production of histories from the material remnants of the past unfolds through the interaction of multiple processes, including existing theoretical frameworks, technoscientific tools and other knowledge-making practices, and processes of ruination and degradation through which we make knowledges from what remains. The work that follows in each of these chapters reflects my ongoing efforts to draw attention to some of the material residues and bodily knowledges that get left behind or excluded by the epistemological limits and power dynamics of population genetics and epigenetics. It is the

beginning of a wish to occupy the lab with desires to know bodies otherwise. Thus, the underlying concerns of this dissertation revolve around new ways of attending to what bodies remember when bodily knowledges are lost, forgotten, ruined, or erased. What material traces and bodily memories survive and what subaltern histories do they know? What meanings are bodies inscribed with and what meanings do they inscribe in the world? How might turning attention to certain subjugated and unruly recollections of matter disrupt received narratives in science? Using a postdisciplinary[1] approach, this dissertation combines genetic research with emerging scholarship from decolonial theory, material and indigenous feminisms, queer theory, and the situated knowledges of white trash people to elucidate the biological and social forces shaping human bodies, as well as the sociopolitical factors that influence how we make knowledge about bodies and histories in settler colonial states.

This research is deeply informed by emergent philosophies in queer and feminist materialisms[2], which have offered productive ways to rethink the connections between nature and culture. Questions of materiality, particularly those which pertain to bodies and biology, have long been volatile subjects within feminist and queer theory (Alaimo and Hekman 2008). As a result, much of queer and feminist scholarship has turned toward social constructivist models to critique the heteropatriarchal power relations embedded within western discursive practices. While this so called "discursive turn" in queer and feminist scholarship has dealt profound blows to scientific objectivity and its authorities

[1] I use the term "postdisciplinary" in place of "interdisciplinary" or "transdisciplinary". Rather than referring to a process that involves working from within, across, or between disciplinary divides, the term "postdisciplinarity" refuses to divide various knowledge-making practices into conventional academic disciplines, and highlights a process in which multiple capacities for making knowledge are brought together in spite of disciplinary norms.

[2] The term "materialism" refers to philosophies of materiality. As used here, it refers to scholarship that deals with physicality, corporeality, or matter as a locus for producing knowledge or academic discourse.

over "bodies" and "natures", less attention has been given to the need for active reconfigurations of these natures as queer and feminist imperatives. Without such reconfigurations, questions of materiality can often remain uncontested ground for the reproduction of racist, heterosexist, and patriarchal infrastructures. In addition, social constructivist models that lack concern for materialities can lead us into relativistic worlds through which it is difficult or impossible to reckon with the bodily consequences of power and its abilities to reshape human and non-human life.

Over the last decade, multiple genealogies of scholarship have coalesced around, and offered alternatives to, the antibiologism that has long characterized much of queer and feminist theory (Barad 2007, Alaimo and Hekman 2008, Mortimer-Sandilands and Erickson 2010). The so called "material turn" (Alaimo and Hekman 2008) in queer and feminist scholarship involves many efforts to take up questions of materiality and its embeddedness within discursive practices as grounds for queer and feminist engagement. As outlined by Stacy Alaimo and Susan Hekman in their influential volume "Material Feminisms", "feminist theorists of the body want definitions of corporeality that can account for how the discursive and the material interact in the constitution of bodies" (Alaimo and Hekman 2008). Similarly, Karen Barad notes that feminist theorists must "move conversations…beyond the mere acknowledgment that both material and discursive, and natural and cultural, factors play a role in knowledge production by examining how these factors work together, and how conceptions of materiality, social practice, nature, and discourse must change to accommodate their mutual involvement" (Barad 2007). While social constructivist interventions have shown us that science operates as a cultural process and a system of knowledge production that too often reifies sociopolitical norms, engagement with emerging queer and feminist materialisms holds

radical potentials for transforming what science can mean and reimagining the worlds that it helps to materialize.

In my dissertation research, the most influential scholarship for answering these calls is that which I find to be in the spirit of Bruno Latour's formulation of the "new settlement", Donna Haraway's concepts of "material-semiotics" and "naturecultures", and Karen Barad's intervention on "agential realism", theoretical frameworks which represent significant efforts to elucidate the interstices between nature and culture without privileging one over the other. In particular, Barad's notions of "intra-action", in which bodies and knowledge, materiality and discursivity, must be understood as part of one, co-constitutive process, underlies much of the work unfolded in this dissertation, and in future work that will come out of what I have learned in producing it. The broadest goal of my work has been to move beyond seeing the body as a fixed, immutable, or passive referent that exists outside the influence of power, demonstrating instead that bodies are a "shifting entanglement of relations" between social and biological forces (Barad 2007). In other words, bodies emerge through the interactions of nature and culture. Bodies are always already bodies-in-context. Bodies are irreducibly worldly happenings. Thus, my goal is to engage matter not as a "thing" but as a "doing", a "congealing of agency" (Barad 2007). Bodies and histories are not simply "out there", they are produced through discursive practices which serve to bring certain kinds of bodies and histories into being to the exclusion of others. Because of this, scientific apparatuses are not simply neutral probes of "nature" (Barad 2007). Rather, they are always loaded with certain conditions of possibility and meaning.

However, while previous new materialist scholarship has retheorized the interactions between social and biological forces, these insights must still be brought to

bear on scientific practices themselves. Therefore, building on insights from queer, material, and indigenous feminists, this dissertation aims in part to take on the lab as a site of political transformation, to claim empirical ground for the intra-action of bodily and sociopolitical forces in genetics and epigenetics. Each of these chapters, in one form or another, is intended to provide anthropological case studies in queer and feminist materialisms, moving emergent gender scholarship directly into sites of empirical inquiry by investigating how systems of power shape bodies. My referent is neither bodies nor power systems in isolation, but rather their intra-action, the entwining of bodies into history and of history into bodies. I do not take matter to be some immutable substance for empirical study, but rather I ask, what are the conditions that underlie certain forms of mattering, and what are some of their possible alternatives?

This work involves a layering of multiple insights and approaches from population genetics, epigenetics, paleogenomics, archaeology, and social theory. I consider the "interdisciplinary", or better, "postdisciplinary" spirit of my dissertation to be deeply informed not only by emerging work in queer and feminist materialisms, by the lived experiences and situated knowledges of both bisexual and white trash people. According to Angela Willey, in her *Dreams of a Dyke Science*, "the feminist scientist will not only "be aware" of the interconnectedness of the personal and the political; that awareness will lead to a fundamental transformation of science's very definition. The dream is not for a better science, but for a different one". In ongoing work by myself and Samantha Archer, we are attempting to unfold what queer and bisexual epistemologies can contribute to the political and epistemological transformation of the lab. As many postmodern and material feminists have noted, the male/female divide informs all the dichotomies that ground Western thought, including nature/culture, mind/body, subject/object, self/other,

reason/passion, human/nonhuman, science/fiction, and by extension, all of the disciplinary divides that have severed the natural sciences from the humanities (Alaimo and Hekman 2008). However, such divisions are irreconcilable with queer experience, where gender binaries have never been a given. My dissertation research, then, is in part driven by a situated refusal to abide by certain gendered structures of knowledge. My disciplinary promiscuity reflects a set of queer desires, a movement through the gendered spaces of the academy and beyond the gendered conventions that fracture knowledge about bodies and police how and what we are supposed to know about them. This dissertation reflects certain infidelities to Science, or perhaps, new kinds of fidelity and new kinds of relations with science that entail engagement with multiple simultaneous capacities for knowing bodies.

For me, these interdisciplinary tendencies cannot be disentangled from the situated knowledges and experiences of white trash people, in particular, the experiences of both rural agricultural and urban poor whites in the American south who live and die on the margins of whiteness. Put simply, to be white trash in the academy is to be forced early and often to learn to stand in multiple places simultaneously and to hold different worlds together. To navigate multiple ways of knowing, and to move along multiple axes of privilege and exclusion. It is to learn to live in the mess of contradictions without righteousness, and because of this, to work without the hope of moral absolutes or final answers. To be white trash is to never really leave the material and political mess that you were born into. It means that my interests in violence and power are rooted in certain lived experiences of violence and power, and that my embodied knowledges permeate all the work that I do. It means to understand, first hand, how the emergence of certain kinds of bodies are irrevocably tied to systems of power and exclusion. While these factors are almost certainly not true for everyone who would identify as queer or as white trash, they

are the center from which my own work has unfolded. And while our particular experiences are not the same, these factors are the driving forces for entangling my work with other queer, material, and indigenous feminists among whom I find many kin and with whom I share many common anti-violence, anti-racist, and decolonial interests.

In this dissertation, I work from these intersectional knowledges to elucidate the entanglement of bodily and political forces in the constitution of human bodies. While each chapter uses different narrative styles and methodological approaches, and the theoretical frameworks are not always made visible, the driving force of this work is to engage various systems of power through which matter is congealed. Specifically, I first look at the boundary making practices and power relations through which population genetics materializes certain indigenous bodies and histories to the exclusion of others. Second, I develop and evaluate new methodological approaches for reconstructing epigenetic marks in ancient DNA, contributing to the development of the emerging field of paleoepigenetics. Finally, I use emerging approaches in paleoepigenetics to show how global genomic methylation patterns reflect sociopolitical and environmental changes in the ancient world.

In chapter one, I argue that the early population histories of the Americas have been the primary focus of genetic research with Native American tribes and First Nations peoples, and by comparison, far less emphasis has been placed on more recent histories, including the genetic correlates of settler colonialism. Conventionally, population genetic research with these groups has involved sampling people who trace most or all of their ancestors to the indigenous peoples present in the Americas prior to the arrival of Europeans. Geneticists studying pre-colonial population histories have often excluded tribal and First Nations members who have "mixed ancestries", because their genetic diversity is presumed to confound efforts to reconstruct ancient population history. In this

study, I present new genetic data from a collection of frozen blood samples originally assembled by researchers in the 1990s. Blood samples from people with primarily indigenous ancestors were selected from this collection in the past for population history analysis, while others with non-indigenous ancestors and/or ancestors from multiple indigenous peoples were largely excluded from study. In this chapter, I use tribal and First Nations affiliations instead of excluding samples on previous genetic standards of biological purity. In doing so, I draw on material and indigenous feminisms to investigate the boundary-making practices and conditions of possibility through which genetic research materializes certain indigenous bodies and histories to the exclusion of others.

In chapter two, I develop and evaluate tools for reconstructing cytosine methylation in ancient DNA, contributing to the emerging field of paleoepigenetics. In living populations, epigenetic research has shown that cytosine methylation patterns can be shaped by a variety of lived experiences, including diet and exposure to violence, among other factors. By looking for similar methylation patterns in the DNA of ancient people, it may be possible to reconstruct certain lived experiences in ancient societies. However, because various epigenetic functions operate on different scales, from single cytosines to hundreds, different resolutions of methylation data are required to make varying types of inferences. The goal of this chapter was to evaluate an emerging technique that exploits patterns of post-mortem cytosine degradation to reconstruct genome-wide methylation patterns in five ancient genomes with differing levels of preservation. I found that while this method can be used to reconstruct regional methylation patterns, providing broad insights into how lived experiences shape cytosine methylation at the global genomic level, it cannot be used to characterize the small, functionally-specific epigenetic changes that often accompany differences in diet and exposure to violence. Combining approaches from

biochemistry and bioinformatics, this chapter offers technical insights into the methodological limits of paleoepigenetics, and lays some of the necessary empirical groundwork for the paleoepigenetic case studies I unfold in my final chapter.

In my third and final chapter, I evaluated whether global genomic methylation patterns were associated with sociopolitical and environmental changes in ancient societies. This study focused on the rise and decline of the Wari, one of the earliest expansive state civilizations of the Andes. During the height of Wari culture, bioarchaeological analyses have shown that people in the Wari heartland had good access to carbon-enriched foods, rates of violence were generally low and injuries were non-lethal, and people's exposure to physical trauma was shaped by status, gender, and ethnic differences in Wari society. Following the decline of the Wari state, however, life appears to have worsened for many people across the central Andes. In the former Wari heartland, bioarchaeological evidence shows that many people had decreased access to carbon-enriched foods and there were dramatic increases in the risk of both lethal and non-lethal violence. In this chapter, I reconstructed patterns of cytosine methylation from the remains of people who lived before and after the Wari decline. In doing so, I provide the first evidence that methylation patterns may mirror social and environmental changes in the ancient world, supporting the feasibility of future research on the epigenetic correlates of ancient lifeways. Thus, paleoepigenetics may provide important new tools for assessing how large-scale social, political, and environmental changes can shape human biology.

This dissertation draws together approaches from population genetics, epigenetics, bioarchaeology, social theory, and my own situated perspectives to demonstrate how bodies are co-constituted by the intra-actions of social and material forces. While the latter two chapters are heavily empirical in form, and the theoretical frameworks underlying them

are not made fully visible here, they are nonetheless deeply informed by queer, feminist, and other emerging materialist philosophies. Thus, while the postdisciplinary goals of my ongoing work are not fully realized in these pages, this dissertation lays some of the necessary groundwork on which my future work will unfold. These chapters reflect some of my first steps towards the goal of realizing a different science, and of integrating multiple simultaneous capacities for knowing bodies.

REFERENCES

Alaimo S and Hekman S. 2008. Material Feminisms. Bloomington: Indiana University Press.

Barad K. 2007. Meeting the Universe Halfway: Quantum Physics and the Entanglement of Matter and Meaning. Durham: Duke University Press.

Haraway D. 1992. The Promises of Monsters. Abingdon: Routledge.

Mortimer-Sandilands C and Erickson B, eds. 2010. Queer Ecologies: Sex, Nature, Politics, Desire. Bloomington: Indiana University Press.

Willey A. 2016. Undoing Monogamy: The Politics of Science and the Possibilities of Biology. Durham: Duke University Press.

CHAPTER ONE: In Cold Blood—Genetic Boundary-Making and the Production of Indigenous Histories.

BACKGROUND

While the genetic diversity of Native American tribes and First Nations groups has been shaped by both ancient and contemporary population histories, the ancient population histories of the Americas have received far greater attention than the more recent events of settler colonialism (Kemp and Schurr 2010, O'Rourke and Raff 2010, Raff et al. 2011, Bolnick et al., 2016). As a result, research on indigenous population histories has typically relied on genetic samples from only a subset of people in indigenous communities — namely, only those who trace all or most of their ancestors to the indigenous inhabitants of the Americas prior to the arrival of Europeans. Genetic diversity in these individuals is presumed to reflect population processes that preceded European colonialism, providing insights into more ancient events.

This approach often excludes individuals who, in the parlance of population genetics, are described as "admixed" — individuals who have some combination of indigenous and non-indigenous ancestors. Genetic samples from these individuals have sometimes been collected but not analyzed, and at other times, they have simply not been collected at all. The rationale underlying these sampling practices is that for researchers wishing to study the deep migratory history of the Americas, it is thought to be preferable to exclude all those whose "genetic ancestry" reflects more recent population histories of settler colonialism. Similarly, in recent years, genomic reconstructions of indigenous population histories have relied on statistical "masking" techniques, or computational methods that "hide" the portions of a person's genome that might be inherited from non-indigenous ancestors. These methods have been intended to reduce or eliminate inferences

drawn from “non-indigenous” portions of a person’s DNA to avoid confounding conclusions about pre-colonial histories (Reich et al. 2012, Rasmussen et al. 2015, Raghavan et al. 2015) — although it is important to note that the “indigenous” portions of a person’s DNA may have also been reshaped by events in the colonial era and might not provide a perfect window into pre-colonial times either. In other words, indigenous peoples did not suddenly cease to move after the arrival of Europeans, quite the contrary.

Notably, individuals have also sometimes been excluded from genetic analysis because they have ancestors from multiple indigenous groups. The ostensible “problem” with sampling these individuals is not genetic exchange between indigenous and non-indigenous peoples, but rather genetic exchange between various tribal or First Nations groups. Such genetic exchange conflicts with assumptions of population isolation, differentiation, and genetic purity that underlie many conventional population genetic models, and individuals with multiple tribal or First Nations affiliations complicate the process of scientific classification, so geneticists have sometimes found it easier to simply ignore their existence.

As a result of these approaches, much has been learned about the initial peopling of the Americas, but less consideration has been given to (1) the population dynamics after the initial peopling of the continents, (2) the genetic correlates of settler colonialism resulting from disease, warfare, genocide, and forced relocations, and (3) the dramatic growth of Native American and First Nations populations beginning in the 20th century. These sampling practices have also led to an incomplete understanding of genetic diversity in indigenous communities today, because some people who are socially recognized as community members have been excluded from genetic analysis. In recent years, a small number of studies have begun to address some of these issues (e.g., Bolnick et al. 2003,

Wang et al. 2004, Bolnick et al. 2006, Hunley and Healy 2011, Moreno-Estrada et al. 2013, and Moreno-Estrada et al. 2014, among others). However, the dominant narratives of genetic research in the Americas have largely centered around only certain indigenous bodies, histories, and concerns, to the exclusion of others.

These ongoing labors of inclusions and exclusions, motivated by scientific desires to discern the "pure" from the "mixed" as a way to access and reproduce certain histories over others, have helped shape what bodies and histories count as indigenous in various material, biological, and political terms. What constitutes indigenous (or any other form of) belonging is not given by "nature" or by biological studies of it; indigeneity is not a pre-existing category passively waiting to be described by a geneticist. Rather, as used in genetics, this category is material-discursive, emerging in and through scientific practices where what is understood as "indigenous" — and what is knowable about the genetic diversity of indigenous peoples — is profoundly shaped by the ideals and expectations of largely non-indigenous scientists. For example, romantic notions of indigenous peoples as isolated, pure, exotic, and primitive others have long been used in colonial states as a way to mark indigenous peoples in a world outside modernity. Such assumptions resonate within contemporary genetic sampling and other scientific practices today, where ostensibly pure, genetically isolated people who are presumed to be untouched by colonial processes are studied as relics of a more distant past. Furthermore, as population genetic research has worked to characterize the indigenous body and indigenous past, it has also served to naturalize the specific sociohistorical conditions of settler colonialism in which the terms "indigenous" and "non-indigenous" have come to have particular meanings.

Material feminist philosopher Karen Barad notes that "scientific apparatuses constrain the material conditions of possibility and impossibility of mattering: they enact

what matters and what is excluded from mattering" (Barad 2007). In "Meeting the Universe Halfway: Quantum Physics and the Entanglement of Matter and Meaning", Barad argues that there is an inseparability between objects and the apparatuses that we use to make knowledge about them, and that they rely on each other for their mutual intelligibility. In other words, she suggests that there are no intrinsic properties in "nature" that exist independently of the scientific apparatuses, structures, and systems that are used to describe them, and we must understand all of these aspects as part of one process. Yet scientific apparatuses are not purely deterministic. Rather, certain scientific apparatuses make room for certain kinds of material possibilities, but not others. In the case of population histories in the Americas, for example, the criteria of "biological purity" precludes certain indigenous bodies and histories from surfacing in discourses of population genetics. Because of this, Barad argues, no scientific inquiries are ever neutral. They are always already historical, political, and power-laden, and it is important to be cognizant of how these issues shape the production of scientific knowledge.

In this paper, we follow Barad's and other material feminist scholars' dual usage of "matter" and "materialize". We take these terms to refer to both 1) the power and politics of science and the way it shapes what can be known about bodies, and 2) the various corporealities that science does and does not bring into being. Our goal is to question boundary-making practices in population genetics and the "conditions of possibility" through which some indigenous bodies and histories come to matter in population genetics, while others are forgotten or erased. We want to highlight the apparatuses that produce certain material configurations of indigenous belonging, and work from other social and theoretical frameworks that might enable us to move towards possible alternatives.

This study focuses on a collection of frozen blood samples that were originally acquired by various population geneticists and medical researchers in the 1990s and subsequently curated by David Glenn Smith at the University of California, Davis. The blood samples in this collection were obtained from indigenous people on the basis of their tribal or First Nations affiliation for the purpose of reconstructing population histories. However, some of the collected samples were omitted from previous genetic analysis because they came from research participants who reported having non-indigenous ancestors or affiliations with multiple tribes and/or First Nations groups. These decisions indicate that scientists have used different criteria for genetic sampling during the various stages of research. Initially, tribal or First Nations affiliations and self-identifications were used to determine whose blood should be collected. Researchers then used blood quantum data and self-reported ancestry information to select the fraction of samples deemed relevant to a particular area of population history research. Thus, within population genetics, there have been different criteria for indigenous belonging at different stages of research, which act to negotiate and renegotiate who counts as indigenous between the syringe and the laboratory bench. These negotiations reflect a layered set of decisions on the part of genetic scientists, where what constitutes indigeneity is progressively sifted and reduced by the boundary-making apparatuses of scientific research. In other words, while many people are socially and culturally cohered as indigenous, only certain indigenous bodies come to "matter" within the technoscientific worlds of human population genetics.

Many of the unanalyzed blood samples have remained frozen and unstudied for two decades, and with them, certain bodily knowledges of settler colonialism have become deanimated in cold blood, deemed irrelevant to dominant scientific discourses about indigenous population history. This biological tissue collection therefore represents an

opportunity to explore how scientific boundary-making practices have shaped knowledge about indigenous DNA, bodies, and histories. To this end, we undertook a genetic analysis of some of the unstudied blood samples to help elucidate the consequences of sampling decisions in previous scientific studies of Native American and First Nations population history. We sought to evaluate how our understandings of genetic diversity and population histories change when we reorient modes of knowledge production in science, privileging tribal and First Nations belonging rather than excluding samples that do not fit within the criteria for inclusion in ancient migrations research, or within traditional scientific definitions of purity.

We therefore obtained and analyzed DNA from frozen blood samples that had been collected from self-identified tribal and First Nations members, but excluded from past studies because they reported having ancestors who were either non-indigenous or from multiple indigenous groups. We analyzed the maternally-inherited mitochondrial DNA (mtDNA) and, for individuals with Y chromosomes, the paternally-inherited Y-chromosome DNA (NRY). We also obtained mtDNA data generated by other researchers who collected samples from Native American tribes and First Nations groups without the use of specific "ancestry" criteria (Schroeder et al. 2011, Hughes et al. 2016). We then assessed genetic diversity patterns across four geographical regions in North America and compared them to the diversity estimates that had been previously reported for those regions. Our results demonstrate that sampling criteria have a significant effect on the patterns of genetic diversity and population histories that can be reconstructed. These results more fully illuminate genetic diversity among contemporary Native American tribes and First Nations groups, and highlight potential genetic correlates of recent colonial histories that have been vastly understudied in North America. Thus, our study

demonstrates how the boundary-making apparatuses of genetic research have helped shape who counts as indigenous and what histories are made visible in human population genetics. Our findings also draw further attention to critical distinctions between how non-indigenous scientists and various indigenous peoples negotiate issues of kinship, identity, and group belonging.

MATERIALS AND METHODS

Samples for Genetic Analysis

We selected 54 blood samples for analysis that had previously been collected from self-identified tribal members but excluded from past studies because they reported having non-indigenous ancestors and/or affiliations with multiple indigenous groups (Lorenz and Smith 1997). These samples were curated at David Glenn Smith's Molecular Anthropology Lab at the University of California, Davis. The sampled individuals provided informed consent for population genetic analyses, and approval for human subjects research was granted by the University of Texas at Austin (IRB protocol 2012-05-0105) and by the University of California, Davis (IRB protocol). To increase our sample size, we also included genetic data from 21 individuals from California and 101 individuals from British Columbia generated by other researchers (Schroeder et al. 2011, Hughes et al. 2016). Individuals were sampled from the California and British Columbia groups on the basis of tribal and First Nations affiliation, without strict requirements for "genetic purity".

Because some indigenous groups are concerned that population genetic studies could have potential ramifications for federal recognition status and issues of legal sovereignty, we conducted our analysis at the regional level, rather than at the tribal or First Nations level, in order to maintain anonymity as much as possible. Based on the geographic locations and culture histories of tribal and First Nations communities, we grouped samples into four geographical regions across North America: Southeast (n=21), Northern Plains (n=30), West (n=24), and Pacific Northwest (n=101) (Figure 1.1). Genetic data from previous studies of these geographical regions were obtained from published articles as well as an unpublished dissertation (n=228) (Lorenz and Smith 1997, Kaestle 1998, Smith et al. 1999, Malhi et al. 2001, Bolnick and Smith, 2003, Bolnick et al. 2006). Including

both the newly collected and previously reported datasets, 404 genetic samples were analyzed in this study altogether.

DNA Extraction and Genetic Analysis

For samples obtained from the frozen blood collection, DNA was extracted from 100 µL of serum using the DNeasy Blood and Tissue kit (Qiagen), following the manufacturer's protocol except in the final step, where DNA was eluted in 50-100 µL of Buffer EB. Negative control extractions (blanks) were included to monitor for possible contamination during all extractions. Following DNA extraction, we used the polymerase chain reaction (PCR) to amplify a 650 base pair (bp) fragment of mtDNA, including the first hypervariable region (HVR1), following Kemp et al. (2010). Mitochondrial DNA is inherited solely from one's mother. Because it traces a single line of ancestors (one's matriline), mitochondrial DNA sheds light on maternal relatedness and population movements, and has been widely studied in human migrations research. PCR products were submitted to the DNA Sequencing Facility at the University of Texas at Austin for purification and sequencing. Sequences were analyzed using Sequencher v. 5.3, and were aligned to the Cambridge Reference Sequence to identify sequence differences using a custom function in the [R] statistical environment. Mitochondrial haplotypes (specific mtDNA sequences) and haplogroups (clusters of related haplotypes that share some mutations because they are descended from a common ancestor) were determined via MitoTools, using the PhyloTree mtDNA database (build 17).

We also analyzed a length dimorphism in the amelogenin gene to detect the presence of X and Y chromosomes in each sample (following Sullivan et al. 1993). This method targets a portion of the first intron of the amelogenin gene, which is present in the homologous regions of the X and Y chromosomes. Because the allele on the X

chromosome is 6 bp shorter than the allele on the Y chromosome (due to a deletion), PCR amplification of this locus yields a single amplicon for people with only X chromosomes, but two amplicons (6 bp different in length) for people with X and Y chromosomes. We assessed amplicon sizes using gel electrophoresis with 6% polyacrylamide gels. Because the blood samples we tested were two decades old and the DNA had somewhat degraded over time, amelogenin assays were conducted twice for some samples to control for allelic dropout, or the random failure of one allele to amplify during PCR. If a Y chromosome was detected on the first assay, the sample was not repeated. However, if only X chromosomes were detected on the first assay, the samples were tested a second time to assess the potential for allelic dropout and to confirm that only X chromosomes were present. Results from sample NP23 (Table 1.2) provide an example of allelic dropout in this study, where the first PCR failed to detect the presence of a Y chromosome allele. Since there are many possible combinations of sex chromosomes in humans and it is not possible to determine precisely how many X or Y chromosomes are present using the amelogenin approach, we do not report genotypes as simply XX or XY. In addition, because combinations of sex chromosomes may or may not correspond with a person's sex or gender identity, we avoided the conventional assignment of "male" and "female" using amelogenin tests. Instead, if no Y chromosome was detected after two assays, the consensus of amelogenin runs was determined to be "X only". For samples that yielded X chromosomes on the first run but re-amplification was not successful, no consensus was determined. Finally, if a Y chromosome was detected in either of the two runs for any sample, the consensus of the amelogenin runs was determined to be "X and Y".

For samples from individuals with Y chromosomes, we analyzed 23 short tandem repeats (STRs – short repeated sequences of DNA that vary in the number of repeats in

each individual) found only on the paternally-inherited portion of the Y chromosome (known as the non-recombining region of the Y chromosome, or NRY). Because the NRY is inherited only from one's father, NRY analysis has been used extensively in population history research to assess paternal relatedness, patrilineal descent, and population movements, similar to how mtDNA is used to study maternal relatedness and matrilineal descent. STR profiles for each sample were generated using the PowerPlex Y23 System (Promega), following the manufacturer's protocol. Multiplexed PCR products were submitted to the DNA Sequencing Facility at the University of Texas at Austin for fragment analysis to determine the number of repeats at each Y-STR. Y chromosome haplotypes were defined as the combination of alleles present at the 23 STR loci analyzed, and Y chromosome haplogroups were determined from STR profiles using the online haplogroup predictor tools available from Whit-Athey and the Y-Chromosome STR Haplotype Reference Database (YHRD), with the area selection field set for equal priors. As some of the blood samples had degraded over time, STRs amplified sporadically and we were only able to determine paternal haplogroups and haplotypes with >70% confidence for some of the Y chromosome-bearing individuals.

Statistical Analysis

Haplogroup and haplotype frequencies for each geographical region were calculated for both the mtDNA and NRY datasets in the [R] statistical environment (R Core Team). Newly collected haplotype data were then combined with previously published data to create composite datasets for each of the four geographical regions. For mtDNA data, all sequences were trimmed to the 294 nucleotides (nucleotide positions 16,069-16,362) sequenced in all samples (i.e., those sequenced in this study and those

sequenced in previous studies). Nucleotide position (np) 16183 was excluded from analysis because it is not independent of a C mutation at np 16189 (Bolnick and Smith 2003).

For each of the four geographical regions in North America, we calculated indices of genetic diversity for mtDNA sequences, including nucleotide, haplotype, and haplogroup diversity, in Arlequin v. 3.5 (Excoffier and Lischer, 2010). These indices summarize the average number of differences between sequences at each nucleotide position, the probability of randomly selecting two different alleles from the population (a measure of allele richness), and the probability of randomly selecting two different haplogroups from the population (a measure of lineage richness), respectively. The higher each of these indices are, the greater the genetic diversity that is present in the population. We also calculated the same indices based only on the previously published sequences for each geographical region and compared these to our composite datasets (Lorenz and Smith 1997, Kaestle 1998, Smith et al. 1999, Malhi et al. 2001, Bolnick and Smith, 2003, Bolnick et al. 2006).

To detect any statistically significant differences in measures of genetic diversity, we compared estimates from the previously published data to estimates from our composite dataset for each geographical region using paired t-tests with a Holm-Bonferroni correction for multiple comparisons (Holm 1979). Fisher's exact tests of population differentiation were also performed in Arlequin v 3.5 to evaluate whether the addition of individuals with non-indigenous ancestors or multiple tribal or First Nations affiliations resulted in any significant changes in Native American regional genetic diversity. For NRY data, we calculated haplogroup frequencies for our composite dataset and compared them to previous studies that had either limited analysis to individuals with mainly indigenous ancestors (Bolnick et al. 2006) or included all tribally affiliated people in the analysis

(Hughes et al. 2016). Taken together, these comparisons allowed us to assess the consequences of scientific sampling decisions on measures of genetic diversity by adding samples into the analysis that had previously been excluded.

To evaluate spatial trends in the indices of genetic diversity for mtDNA sequences, we used three linear regression analyses to assess relationships between geography and either haplogroup diversity or the proportion of genetic sequences shared with non-indigenous peoples. For each region in this study (East, Northern Plains, West, and Pacific Northwest), we selected the center of the geographical range where each tribal or First Nations group was located at the time of European colonization and calculated the linear distances in miles starting from the Southeast (0 miles away) to the Pacific Northwest (2245 miles away). We then regressed these distances against each measure of genetic diversity for both previously published datasets and our composite datasets.

For mtDNA and NRY hapotypes not found among the indigenous peoples of North America prior to the arrival of Europeans (Tamm et al. 2007), we used GenBank's Basic Local Alignment Search Tool (megablast) to search human genetic databases for highly similar DNA sequences. The geographic location of the sequences with highest genetic affinity and frequency were used to identify the most likely population with which indigenous people share either maternal or paternal ancestors. However, while the predicted populations are the *most likely* source, these lineages may have also been brought to the Americas from populations where they are found at lower frequencies, from regions where they are not present today but where they were present earlier during U.S. colonization, or from a geographic area or population which has not yet been sampled, and thus is not represented in public genomic databases for comparison.

RESULTS

Mitochondrial DNA sequences were generated for all 54 frozen blood samples (Table 1.1). While we had little difficulty amplifying mtDNA from these samples, as it is present in many copies in each cell, single-copy nuclear loci on sex chromosomes, including the amelogenin and Y-STR loci, amplified with more variable success. This pattern of amplification was not surprising, as the blood samples used in this study were collected more than 20 years ago and many had degraded over time. Of the 54 samples yielding mtDNA, we were able to genotype the amelogenin length dimorphism in a total of 34 (63%) individuals. We determined that 15 (44%) of these individuals carried X and Y chromosomes, while 19 (56%) individuals carried only X chromosomes (Table 1.2). Y chromosome STR data (ranging from 1-16 STRs per sample) were obtained for 8 (53%) of the 15 Y chromosome-bearing samples (Table 1.3). Six of these 15 samples (40%) yielded enough STR loci to estimate paternally-inherited NRY haplogroups with greater than 70% probability (Table 1.3).

The 54 mtDNA sequences generated here were combined with 122 sequences recently collected by other researchers (Schroeder et al. 2011, Hughes et al. 2016) and with previously published studies of the frozen blood collection (Lorenz and Smith 1997, Kaestle 1998, Smith et al. 1999, Malhi et al. 2001, Bolnick and Smith, 2003, Bolnick et al. 2006). Indices of genetic diversity for this composite dataset, and for the previously published sequences from each geographical region, are given in Table 1.4. Notably, when all tribal or First Nations-affiliated individuals were included in the genetic analysis, rather than excluding individuals with ancestors who were either non-indigenous or from multiple indigenous groups, there are important differences in estimates of genetic diversity for all four geographical regions (Tables 1.5 and 1.6). Nucleotide diversity estimates are lower in

the composite datasets for the Northern Plains and the West, but greater in the composite datasets for both the Southeast and the Pacific Northwest. However, pairwise t-tests indicate that differences between current and previous measures of nucleotide diversity were not statistically significant ($p \geq 0.94$, Table 1.5). All measures of haplotype and haplogroup diversity are greater in the composite datasets for the four geographical regions tested—especially in the Pacific Northwest, where haplotype diversity increased by 12% and haplogroup diversity increased by 57%. Pairwise t-tests indicate that the differences between previous and current measures of haplotype and haplogroup diversity are highly statistically significant for all four geographical regions ($p<0.001$, Table 1.5).

Haplotypes and haplogroups inherited from non-indigenous ancestors are more highly represented in our analysis than in previous studies, more fully reflecting settler colonial population histories (Table 1.7 and 1.8, respectively). Mitochondrial haplogroups A2, B2, C1, D1, and X2a are the maternally-inherited haplogroups commonly found among Native Americans and First Nations peoples prior to the arrival of non-indigenous people, and they were the ones identified by previous genetic studies of these populations. In contrast, our study identified low frequencies (3-6%) of mtDNA haplogroups H, I, J, and L in the indigenous communities—sequences which reflect genetic exchange with non-indigenous people who came or were forcibly brought to the Americas from Europe and Africa (Table 1.1). For the paternally-inherited Y chromosome lineages, a similar pattern is seen. While previous studies have shown that some members of Native American tribes and First Nations share paternal ancestors with non-indigenous populations (Bolnick et al. 2006), a greater proportion of these haplogroups are detected when tribal or First Nations affiliation is used in lieu of specific "genetic ancestry" requirements (Tables 1.3 and 1.8). These results show that both tribal and First Nations communities in North America share

some paternal ancestors with populations who came to the Americas from Europe, Africa, or elsewhere in the last five centuries. Notably, the frequency of these paternal lineages is much higher than the frequency of maternal lineages inherited from non-indigenous ancestors over the same time period, reflecting a marked asymmetry in the proportion of paternal and maternal ancestors shared with non-indigenous populations. This pattern is consistent with previous studies of gender-biased genetic exchange in the Americas (for example, Bolnick et al. 2006).

Strikingly, some measures of genetic diversity exhibit spatial patterning across the four geographical regions included in this study. While spatial assessments were not possible with the NRY data due to limited sample sizes, linear regression analyses show that both mtDNA haplogroup diversity and the frequencies of mtDNA haplogroups shared with non-indigenous populations gradually decline from the Southeast to the Northwest (Figures 1.2 and 1.3). Our composite dataset (including all tribally- or First Nations-affiliated people) identified a stronger relationship with East-to-West geography (Figure 1.2B, R^2=0.63) than did our meta-analysis of previously published datasets that excluded some community members (Figure 1.2A, R^2=0.46), though neither relationship was statistically significant ($p \geq 0.13$). However, the proportion of mtDNA haplogroups shared with non-indigenous populations showed a strong and statistically significant westward decline from Southeast to Northwest (Figure 1.2C, R^2=0.95, p=0.016).

DISCUSSION

Genetic Sampling, Boundary-making, and the Production of Indigenous Histories

By reconsidering the criteria used to select samples for genetic studies with tribal and First Nations groups, this study illuminates the consequences of scientific boundary-making practices, showing how the power-laden politics of inclusions and exclusions have helped shape knowledge about indigenous bodies, communities, and histories. When we use the criteria of tribal and First Nations membership as entry points for genetic research, rather than limiting analysis to the subset of individuals who facilitate ancient migrations research, a different understanding of genetic diversity emerges—one that reflects at least three aspects of settler colonial histories in the Americas that had been rendered partially or fully invisible by earlier studies. First, our results demonstrate that the genetic composition of Native American and First Nations communities is more diverse than previously suggested. We identified both mtDNA and Y chromosome haplogroups among tribes and First Nations groups that trace to non-indigenous ancestors and had not been identified in previous studies of this blood collection. We also found that the frequency of Y chromosome lineages inherited from non-indigenous ancestors is much higher than the frequency of mtDNA lineages from such ancestors. This marked asymmetry is consistent with both scientific and oral histories of settler colonial expansion in North America, which was largely male driven, and likely reflects layered histories of sexual violence, partnership, intermarriage, and other relations. While this pattern has been described in previous studies (for example, Bolnick et al. 2006), we show that patterns of patrilineal genetic diversity – and genetic diversity more broadly among tribes and First Nations groups – have been more influenced by settler colonialism than previous studies suggested.

Second, our meta-analysis of previously published genetic data indicates that previous studies could only detect a weak relationship between geography and haplogroup diversity. This relationship is much stronger when all tribal and First Nations affiliated people are included in the analysis, showing a more pronounced decline in haplogroup diversity across the landscape from Southeast to Northwest. Most importantly, we found that the fraction of mtDNA haplogroups that trace to non-indigenous ancestors is strongly and significantly shaped by geographic distance from the Southeast, where European colonialism had many of its earliest impacts in North America. This genetic pattern had been rendered invisible by the boundary-making apparatuses of science that shaped earlier analyses of this and other sample sets. To our knowledge, no previous study has identified an east-to-west decline in mtDNA haplogroup diversity or the fraction of mtDNA that tribes and First Nations trace to non-indigenous ancestors. While our findings are somewhat limited by sample size (n=404) and our focus on a small region of mtDNA, these patterns may reflect the spatial and temporal correlates of European colonialism as settlers expanded westward across the continent over the last five centuries. Many of the earliest colonies were established in eastern North America, so greater haplogroup diversity and mtDNA tracing to non-indigenous ancestors in the Southeast may reflect the longer duration of colonial settlement and history of interaction between indigenous and non-indigenous peoples in this region. Alternatively, these patterns might reflect differences in regional colonization processes and/or shifting patterns of indigenous resistance as settlers expanded across the continent. Further studies are needed to distinguish among these possibilities and better elucidate the genetic correlates of settler colonialism in North America.

Finally, when we used our composite dataset to identify the most likely non-indigenous populations with which indigenous people share maternal or paternal ancestors, we found that tribal and First Nations groups share some ancestors with populations living in Europe and Africa. These inferences are based on the geographic region(s) where each haplogroup is found at the highest frequency in *contemporary*, previously studied populations, and thus are not definitive ties to specific ancestral populations. Nevertheless, these patterns of genetic diversity are consistent with – and clearly reflect – the histories of European settler colonialism and the West African slave trade in the Americas.

Thus, the picture of genetic diversity in indigenous communities that emerges from this study is one that must be understood as a result of *both* ancient and more recent demographic processes. By privileging tribal and First Nations affiliations over criteria of "biological purity", this analysis helps recover histories and demographic processes that have frequently been overlooked in population genetics, and more fully elucidates the genetic correlates of settler colonialism.

Narratives of Indigeneity in Science and Society

Indigenous belongings, indigenous histories, and indigenous identities are all contested spaces in science. Various productions of indigeneity within population genetics should be recognized as entangled with settler colonial power structures, the legacies of racial science, and the layered politics and legal context of kinship and belonging in indigenous communities. However, the dominant apparatus of genetic research in North America, which has overwhelmingly focused on the "origins" of indigenous people and the ancient peopling of the Americas, has largely constituted indigenous bodies as only those which carry narrowly defined "indigenous DNA". But who has been counted as indigenous in bioscientific terms, and whose identity is otherwise materially and socially

constituted as indigenous, do not always align. Genetic research in North America has too often enacted what matters in terms of deep history, and has excluded histories of settler colonialism, along with their genetic correlates. It has also excluded many people whose identity, regardless of their DNA, is cohered in relation to indigenous peoples. These practices, like the centuries of western science that precede them, too often depict Native Americans in anachronistic terms, as biological and cultural relics of a distant past. Conventional population genetic histories in the Americas produce and authenticate certain kinds of indigeneity by privileging "genetic ancestries" over social affiliations, shaping who counts as indigenous in terms of ancient patterns of genetic diversity. Such scientific narratives have long been deployed as a way to mark others as being in a world outside of colonial modernity, and are the foundation upon which settler claims to indigenous belonging have unfolded through direct-to-consumer "genetic ancestry" tests in recent decades (TallBear 2013).

Such claims highlight the distinctions between various modes of defining who is indigenous. Non-indigenous scientists and settler communities have long defined indigeneity in narrow biological and anachronistic terms, characterizing indigenous peoples as ancient and isolated others – unchanging relics of the past. Increasingly articulated in the form of "genetic ancestry", such delineations of indigenous belonging emphasize biological origins and genetic relations, and differ substantially from the understandings of belonging prevalent in many indigenous communities (TallBear 2013). Many indigenous communities recognize belonging as constituting more than one's genetic relations, instead being the product of a complex web of social and cultural ties, political factors, lineal descent, and legal codes (TallBear 2013). In other words, many tribes and First Nations communities see belonging more as a layered set of social and

material connections, and less exclusively as a question of one's "biological origin" (Kolopenuk 2014).

In this context, the idea of "indigenous DNA" that emerges out of human population genetics research deserves further attention. Indigeneity, or any other way that humans constitute identity and belonging, is not given by scientific mediations of DNA, but by one's embeddedness in certain material and social worlds (TallBear 2013). DNA alone therefore cannot be indigenous, and it does not identify who is indigenous and who is not. Thus, while there are indigenous people, there is no meaningful basis for defining "indigenous DNA" as something apart from indigenous personhood[3]. Indigenous people may share genetic sequences in common with non-indigenous people as a result of various settler colonial histories, but that alone does not establish or deny their belonging within indigenous communities. Likewise, non-indigenous people may have DNA sequences that they inherited from an indigenous ancestor, but that alone does not make them "indigenous" (TallBear 2015). Instead, what bodies, identities, cultures, relations, and histories count as indigenous is ultimately a question for various indigenous communities and not for non-indigenous geneticists or other scientists. These issues, guided by the insights of queer, feminist, and indigenous critiques of genomics, shaped the research design employed in this study. Thus, rather than seeking to define what is "indigenous" in genetic terms, the starting point of this study to center the sovereignty of tribal and First

[3] Elsewhere, and in agreement with the work of Kim TallBear (2013), I have noted in autoethnographical writing that white settlers cannot use the DNA that they have inherited from indigenous ancestor(s) as a means for appropriating indigenous identities. Instead, drawing upon the positionalities of white trash people, I have argued that in spite of DNA that I may have inherited from my indigenous ancestors, without specific ties to indigenous communities my identity can only be understood as constituted within the histories and social relations of white trash people in the pastoral and urban south. Similarly, Māori scholar Māui Hudson has used the notion of "taonga" (treasure to be stewarded) to describe how DNA that he inherited from his Scottish ancestor(s) can be understood as appropriated within his Māori identity. In both cases, DNA cannot be understood as something apart from various modes of personhood and social belonging.

Nations groups to define who they are and who is a member of their communities. From there, we worked to recover the various invisibilized political and social processes that shape bodies, and to attend to bodily knowledges that have been hidden or lost in previous studies of population history in the Americas.

The conventional omission of "admixed" members of indigenous communities – those who have ancestors from multiple tribes, First Nations, or non-indigenous populations – reflects a number of power relations that are shaped by the embeddedness of genetic science within settler colonial regimes and their desires to define indigeneity in terms of the entangled concepts of purity, death, and extinction (TallBear 2013). The urgency of ongoing scientific gazes directed at biologically "pure" Natives over those who are increasingly "mixed" emerges from and contributes to the imagined extinction of indigenous peoples and their ongoing histories. This has the effect of reifying biological notions of difference and reinvigorating false perceptions of the "disappearing Native".

Such views hinge on the strict scientific definition of indigenous people through narrow patterns of genetic variation that existed in the Americas in the past, conflating notions of purity and stasis with those of survival. But themes of purity, mixedness, life, and death are unevenly mapped across communities in settler colonial societies. While increasing mixedness is often seen as the end of indigenous peoples, mixedness among settlers is used to support certain idealized notions of the virtuous, liberal, *multicultural* citizen in contemporary settler colonial society (TallBear 2015) – the white settler with "indigenous DNA" through whose body we are ostensibly supposed to witness the final resolution of colonial history, the morally sanctioned possession of indigenous lands, and the emergence of an imagined postracial era. Mixedness, then, simultaneously signals indigenous extinction and settler futures in the colonial consciousness (TallBear 2015).

In this regard, genetics has served the larger political structures of colonialism, where settlers imagine and enact their own identities and entitlements through collective imaginings of settler life and indigenous death (Morgensen 2011, TallBear 2013). However, as scientists and settler societies wrestle with their colonial anxieties about the increasing "mixedness" among indigenous people, indigenous scholars like Chris Andersen have reasserted that notions of tribe and nation, rather than biological purity or mixedness, are those which are foundational to indigenous survival and modes of belonging (Andersen 2014). These issues underscore crucial distinctions between bioscientific and indigenous articulations of belonging. While some avenues of population genetic research have long reproduced themes of indigenous death and facilitated certain possessions of indigenous bodies, identities, and cultures, the genetic diversity of tribal and First Nations-affiliated people seen in this study instead underscore notions of vitality, dynamism, and survival through centuries of settler colonialism.

These concerns have shaped the research design and scientific concepts employed in this study. For example, we avoided employing the population genetics concept of "admixture" wherever possible. Instead, we refer to "genetic exchange", to highlight how DNA sequences are shared between populations without the racializing presumptions that there were ever pure "source" populations. We also emphasize that DNA is inextricably embedded in and shaped by social processes. From these starting points, we have worked to recover the various political and social processes that shape bodies but have been made invisible, and to attend to bodily knowledges that have been hidden or lost in previous studies of population history in the Americas.

Thus, by reanalyzing previously collected genetic samples, we have interrogated the scientific boundary-making practices and "conditions of possibility" through which

certain indigenous bodies and histories have been materialized to the exclusion of others. By privileging tribal and First Nations definitions of who counts as indigenous, we recover recent population histories that have been understudied in population genetics – histories that make the genetic correlates of settler colonialism visible, and that do not presume that indigenous peoples are unchanging relics of the past, but ongoing, dynamic, living communities. Our research further destabilizes the idea of indigenous belonging as something that is biologically constituted, and begins to shift the boundary-making practices of population genetics that conventionally serve to bring only certain indigenous histories, and certain indigenous bodies, into view.

REFERENCES

Andersen C. 2014. Metis: Race, Recognition, and the Struggle for Indigenous Peoplehood. Vancouver: UBC Press.

Barad K. 2007. Meeting the Universe Halfway: Quantum Physics and the Entanglement of Matter and Meaning. Durham: Duke University Press.

Bolnick DA et al. 2006. Asymmetric male and female genetic histories among Native Americans from Eastern North America. Mol Biol Evol 23:2161–74.

Bolnick DA and Smith DG. 2003. Unexpected Patterns of Mitochondrial DNA Variation among Native Americans from the Southeastern United States. Am J Phys Anthropol 122:336–354.

Bolnick DA et al. 2016. Native American Genomics and Population Histories. Annu Rev Anthropol:1–22.

Excoffier L and Lischer HEL. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564–567.

Hughes CE. Genetic structure of First Nation communities in the Pacific Northwest (unpublished).

Kaestle F a. 1998. Molecular evidence for prehistoric native American population movement: the numic expansion. (Doctoral Dissertation).

Kemp BM and Schurr TG. 2010. Ancient and Modern Genetic Variation in the Americas. In: Human Variation in the Americas p 12–50.

Kolopenuk J. 2014. Becoming Native American: Facializing Indigeneity in Canada through Genetic Signification and Subjection. Annual Meeting of the Native American and Indigenous Studies Association. Austin, Texas.

Lorenz JG and Smith D. 1997. Distribution of Sequence Variation in the mtDNA Control Region of Native North Americans. Hum Biol 69:749–776.

Malhi RS et al. 2001. Distribution of mitochondrial DNA lineages among Native American tribes of Northeastern North America. Hum Biol an Int Rec Res 73:17–55.

Morgensen S. 2011. Spaces Between Us: Queer Settler Colonialism and Indigenous Decolonization. Minneapolis: University of Minnesota Press.

O'Rourke DH and Raff JA. 2010. The human genetic history of the Americas: the final frontier. Curr Biol 20:R202–7.

R Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raff JA et al. 2011. Ancient DNA perspectives on American colonization and population history. Am J Phys Anthropol 146:503–14.

Raghavan M et al. 2015. Genomic evidence for the Pleistocene and recent population history of Native Americans. Science 349(6250):1-11.

Rasmussen M et al. 2015. The ancestry and affiliations of Kennewick Man. Nature 523:455-458

Reich D et al. 2012. Reconstructing Native American population history. Nature488:370–4.

Schroeder KB et al. 2011. Biological Continuity in the Central Valley: Evidence from Ancient and Modern Mitochondrial DNA. J Calif Gt Basin Anthropol 31:37–56.

Smith DG et al. 1999. Distribution of mtDNA haplogroup X among Native North Americans. Am J Phys Anthropol 110:271–84.

Sullivan et al. 1993. A Rapid and Quantitative DNA Sex Test – Fluorescence-Based PCR Analysis of X and Y Homologous Gene Amelogenin. Biotechniques 15(4):636-8

TallBear K. 2013. Native American DNA: Tribal Belonging and the False Promise of Genetic Science. Minneapolis: University of Minnesota Press.

Tamm E et al. 2007. Beringian standstill and spread of Native American founders. PLoS One 2:e829.

Table 1.1: Mitochondrial DNA Haplogroups and Haplotypes.

Sample ID	Geographic Region	mtDNA Haplogroup	mtDNA Haplotype
NP1	Northern Plains	C	223T, 298C, 327T
NP2	Northern Plains	H	NA
NP3	Northern Plains	C	172C, 223T, 298C, 325C, 327T
NP4	Northern Plains	A	111T, 223T, 230G, 290T, 319A, 362C
NP5	Northern Plains	A	111T, 192T, 223T, 290T, 319A, 362C
NP6	Northern Plains	X	189C, 223T, 278T
NP7	Northern Plains	C	223T, 298C, 325C, 327T
NP8	Northern Plains	A	111T, 209C, 223T, 290T, 319A, 362C
NP9	Northern Plains	C	223T, 298C, 327T
NP10	Northern Plains	B	179A, 181C, 182C, 183C, 189C, 217C
NP11	Northern Plains	A	111T, 223T, 290T, 319A, 362C
NP12	Northern Plains	A	111T, 192T, 223T, 290T, 319A, 362C
NP13	Northern Plains	A	111T, 223T, 290T, 319A, 325C, 362C
NP14	Northern Plains	A	126C, 129A, 223T, 290T, 319A, 324C, 362C
NP15	Northern Plains	C	223T, 298C, 327T, 362C
NP16	Northern Plains	C	195C, 223T, 298C, 327T
NP17	Northern Plains	C	223T, 294T, 298C, 325C, 327T
NP18	Northern Plains	C	223T, 298C, 327T
NP19	Northern Plains	A	111T, 223T, 290T, 319A, 362C
NP20	Northern Plains	A	111T, 223T, 230G, 290T, 319A, 362C
NP21	Northern Plains	B	182C, 183C, 189C, 217C
NP22	Northern Plains	B	182C, 183C, 189C, 217C
NP23	Northern Plains	D	223T, 303A, 325C, 362C
NP24	Northern Plains	H	354T

NP25	Northern Plains	H	NA
NP26	Northern Plains	A	111T, 223T, 290T, 319A, 362C
NP27	Northern Plains	H	148T
NP28	Northern Plains	B	182C, 189C, 217C
NP29	Northern Plains	C	223T, 298C, 327T
NP30	Northern Plains	H	162G
SE1	Southeast	A	111T, 223T, 290T, 319A, 362C
SE2	Southeast	L	124C, 223T, 278T, 362C
SE3	Southeast	C	223T, 298C, 325C, 327T
SE4	Southeast	J	069T, 126C, 261T
SE5	Southeast	B	153A, 183C, 189C, 217C
SE6	Southeast	A	111T, 223T, 290T, 319A, 362C
SE7	Southeast	C	223T, 298C, 325C, 327T
SE8	Southeast	A	111T, 223T, 290T, 319A, 362C
SE9	Southeast	A	111T, 223T, 249C, 290T, 319A, 362C
SE10	Southeast	I	129A, 223T
SE11	Southeast	A	111T, 223T, 290T, 319A, 362C
SE12	Southeast	A	111T, 184T, 223T, 290T, 319A, 362C
SE13	Southeast	H	NA
SE14	Southeast	A	111T, 223T, 249C, 290T, 319A, 362C
SE15	Southeast	H	304C
SE16	Southeast	A	104T, 111T, 223T, 290T, 319A, 362C
SE17	Southeast	A	111T, 223T, 234T, 250T, 290T, 319A, 362C
SE18	Southeast	A	111T, 223T, 249C, 290T, 311C, 319A, 362C
SE19	Southeast	A	111T, 223T, 249C, 290T, 319A, 362C
SE20	Southeast	B	114T, 179T, 182C, 189C, 217C

Table 1.1 cont.

SE21	Southeast	B	114T, 179T, 182C, 189C, 217C
W1	West	B	183C, 189C, 217C
W2	West	B	111T, 189C, 217C
W3	West	A	111T, 223T, 290T, 319A, 362C

Table 1.1 cont.

mtDNA haplogroups are reported with names that are composed of a combination of letters and numbers. Haplotypes are often reported as sets of DNA differences (mutations) from a particular reference sequence. For example, the mtDNA haplotype "16,223T, 16,298C, and 16,327T" indicates that there are three differences from the Cambridge reference sequence. The first difference is a thymine that is present at the 16,223rd position of the mtDNA, the second difference is a cytosine at the 16,298th position, and so on. In this study, sequence differences are reported from nucleotide positions 16,069-16,362. NA-no differences from the Cambridge reference sequence.

Table 1.2: X and Y chromosomes identified by the amelogenin assay.

Sample ID	First Test	Second Test	Consensus
NP2	X only	X only	X only
NP6	X only	X only	X only
NP7	X only	X only	X only
NP9	X and Y		X and Y
NP11	X and Y		X and Y
NP13	X and Y		X and Y
NP14	X and Y		X and Y
NP15	X and Y		X and Y
NP16	X only	X only	X only
NP18	X only	X only	X only
NP20	X and Y		X and Y
NP21	X and Y		X and Y
NP22	X and Y		X and Y
NP23	X only	X and Y	X and Y
NP24	X and Y		X and Y
NP25	X and Y		X and Y
NP26	X only	X only	X only
NP27	X only	X only	X only
NP29	X only	X only	X only
NP30	X and Y	X only	X and Y
SE2	X only	X only	X only
SE4	X only	X only	X only
SE6	X and Y		X and Y

SE7	X only	X only	X only
SE8	X and Y	X only	X and Y
SE9	X only	X only	X only
SE10	X only	X only	X only
SE11	X only	X only	X only
SE12	X only	X only	X only
SE13	X and Y		X and Y
SE16	X only	X only	X only
SE19	X only	X only	X only
SE20	X only	X only	X only
SE21	X only	X only	X only

Table 1.2 cont.

Table 1.3: NRY Haplotypes and Haplogroups.

Sample	DYS 385a	DYS 385b	DYS 389I	DYS 389II	DYS 390	DYS 391	DYS 393	DYS 437	DYS 438	DYS 448	DYS 456	DYS 458	DYS 481	DYS 533	DYS 549	DYS 570	DYS 576	DYS 635	HG*	F†	Pr‡
NP9	15	16	13	28	23	9	12	14	10	21		19	30	12	12		17	23	**I2a**	29	100
NP13												18									
NP14					23	10	12			22	14		27	11	13	19	16	20	**J1**	43	99.7
NP15						11						17				16					
NP20			14			10	13					16	25	10	11	18	20		**Q**	54	73.2
SE8			14			10	13			19		17	25			18	19	22	**Q**	76	96.7
SE9	14	16	14	30		10		14	11			17							**Q**	82	86.3
SE13			13			11		14	12			17	22			17			**R1b**	73	99.6

STR analyses assess differences in the length of short repeated sequences of DNA. For example, the STR position on the NRY known as DYS385 is made up of 4 nucleotide bases, [GAAA], which are repeated between 7 and 28 times depending on the person. At this STR position, some people have [GAAA]7 while others have up to [GAAA]28, indicating that the "GAAA" sequence is repeated 7 and 28 times in a row, respectively.

*HG: Haplogroup assignment

†F: Whit-Athey predicted fitness score

‡Pr: Probability of haplogroup prediction(%)

Table 1.4: Indices of mtDNA Genetic Diversity.

Region	Sample Set	N	Nucleotide Diversity (π)	Haplotype Diversity	Haplogroup Diversity
Southeast	Bolnick and Smith 2003	63	0.0195	0.9601	0.7394
	Current Study*	84	0.0196	0.9745	0.7516
Northern Plains	Lorenz and Smith 1997 Smith et al. 1999 Malhi et al. 2001	80	0.0247	0.9766	0.6696
	Current Study*	110	0.0225	0.9852	0.7063
West	Lorenz and Smith 1997 Kaestle 1998	66	0.0188	0.9534	0.6876
	Current Study*	90	0.0186	0.9678	0.7051
Pacific Northwest	Lorenz and Smith 1997	19	0.0165	0.8421	0.3801
	Current Study*	120	0.0184	0.9471	0.5986

*Composite dataset including previously published and newly collected HVR1 data

Table 1.5: Paired T-Tests Between Previous and Current Estimates of Genetic Diversity for each Geographic Region.

	Holm-Bonferroni corrected p-values		
	Nucleotide Diversity	**Haplotype Diversity**	**Haplogroup Diversity**
East	1.00	<0.0001	<0.01
Plains	0.94	<0.0001	<0.0001
West	1.00	<0.0001	<0.001
NorthWest	1.00	<0.0001	<0.0001

Table 1.6: Fisher's Exact Test of Population Differentiation.

		East		Plains		West		Northwest	
		Previous	Current	Previous	Current	Previous	Current	Previous	Current
East	Previous	0.0000							
	Current	0.0327	0.0000						
Plains	Previous	0.0316	0.0245	0.0000					
	Current	0.0272	0.0202	0.0191	0.0000				
West	Previous	0.0433	0.0360	0.0350	0.0305	0.0000			
	Current	0.0360	0.0289	0.0278	0.0235	0.0393	0.0000		
Northwest	Previous	0.0939	0.0856	0.0846	0.0794	0.0973	0.0889	0.0000	
	Current	0.0466	0.0394	0.0384	0.0339	0.0499	0.0427	0.0995	0.0000

Table 1.7: Mitochondrial Haplogroup Frequencies.

Haplogroup	Southeast		Northern Plains		West		Northwest	
	Previous	*Current*	*Previous*	*Current*	*Previous*	*Current*	*Previous*	*Current*
A	0.32	0.36	0.50	0.45	0.05	0.04	0.79	0.60
B	0.30	0.26	0.15	0.15	0.42	0.42	0.11	0.03
C	0.25	0.23	0.23	0.25	0.27	0.21	0.05	0.16
D	0.13	0.10	-	0.01	0.26	0.28	0.05	0.13
X	-	-	0.13	0.10	-	-	-	0.07
Other	-	0.06	-	0.05	-	0.04	-	0.03

Table 1.8: NRY Haplogroup Frequencies.

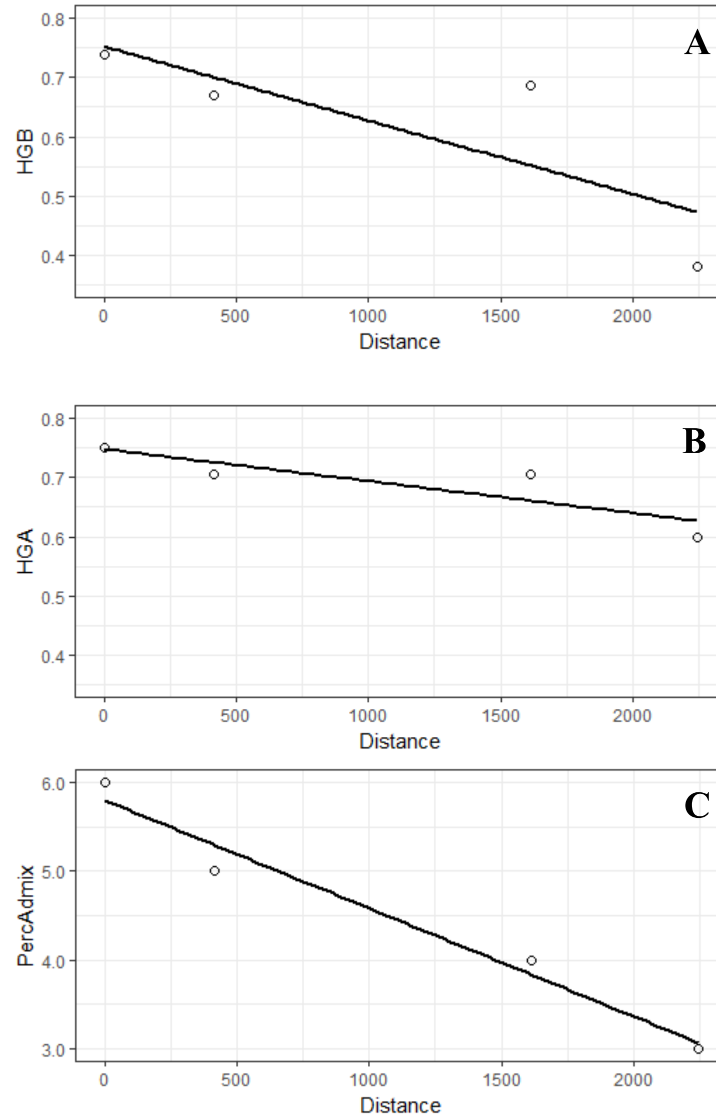
Haplogroup	Bolnick et al. 2006* (n=98)	Hughes et al. 2016† (n=61)	Current Study‡ (n=165)
Q	0.59	0.30	0.48
C	0.02	0.06	0.04
Other	0.39	0.64	0.48

*Data includes NRY haplogroups reported for the Southeast region only
†Data includes NRY haplogroups reported for the Pacific Northwest region only
‡Composite dataset including previously published and newly collected NRY data

Figure 1.1: Geographic Regions and Samples Sizes.

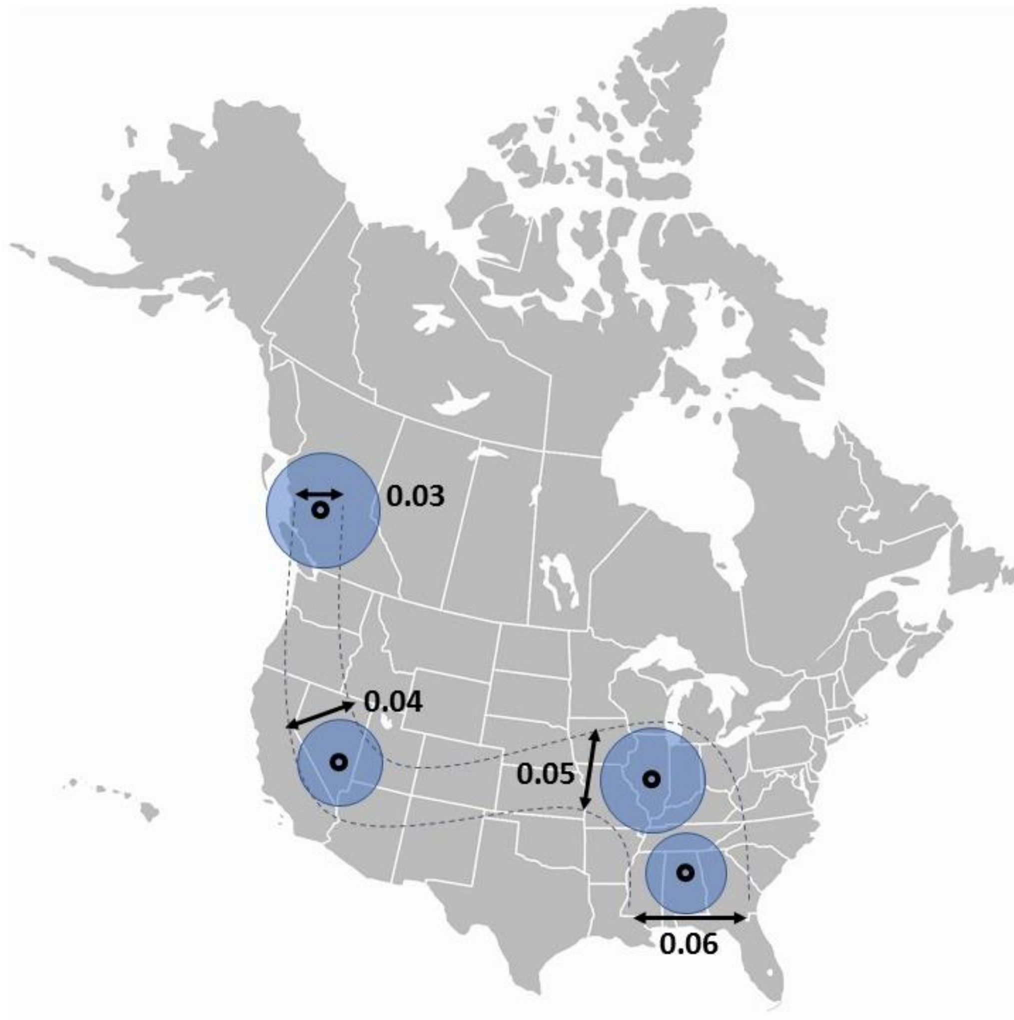
Small black circles indicate midpoints for the four geographic regions included in this study – Southeast, Northern Plains, West, and Pacific Northwest. Blue shaded circles are proportional to the relative sample sizes in the composite dataset of mtDNA sequences, which includes both newly sequenced and previously published genetic data.

Figure 1.2: Linear Regression Analyses of Haplogroup Diversity, the Fraction of Haplogroups from Non-Indigenous Ancestors, and Distance.



Linear regression analyses of genetic diversity indices as a function of distance starting from the Southeast (0 miles) to the Northwest (2245 miles). **(A)** Haplogroup diversity estimates calculated from previously published mtDNA sequences. **(B)** Haplogroup diversity estimates calculated from the composite dataset of newly collected and previously published mtDNA sequences. **(C)** The fraction of mtDNA lineages (%) among tribal and First Nation affiliated people from the composite dataset that is shared with non-indigenous settler populations.

Figure 1.3: Westward Decline in Proportion of mtDNA Settler Populations.



Small black circles indicate midpoints for the four geographic regions included in this study. Blue shaded circles are proportional to the mtDNA sample sizes available for each region. The width between the dashed lines, highlighted at four key points by black arrows, indicate the relative frequency of mtDNA haplogroups that are shared with non-indigenous people. These frequencies decrease across the continent from Southeast to Northwest, possibly mirroring the spatial and temporal history of westward settler colonial expansion.

CHAPTER TWO: Evaluating Deamination-based Approaches for Inferring DNA Methylation: Insights from Five Ancient Genomes.

BACKGROUND

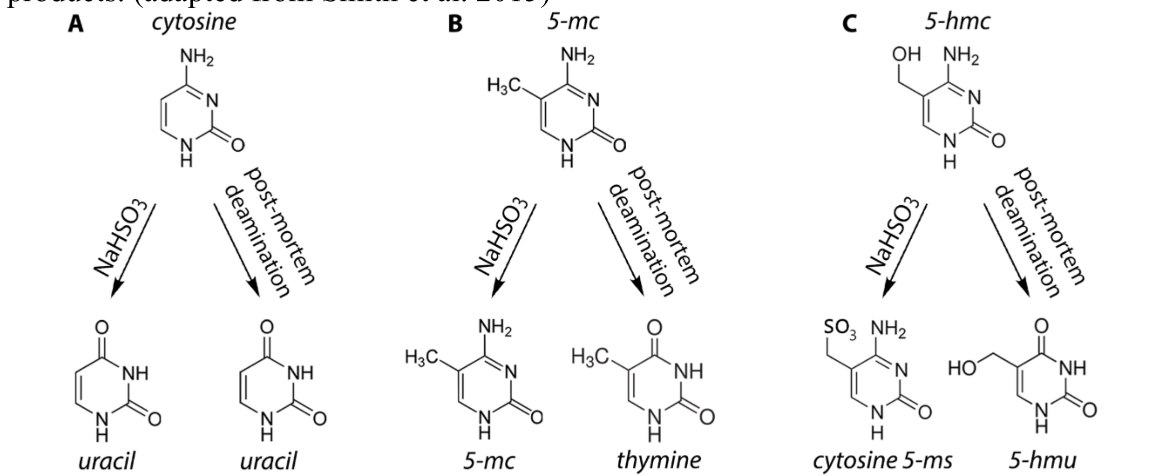
Paleoepigenetics is an emerging area of ancient DNA (aDNA) research that reconstructs epigenetic modifications to DNA from ancient organisms and evaluates their environmental and evolutionary significance. In recent years, a small but growing number of studies have shown that cytosine methylation patterns can be successfully recovered from aDNA (reviewed in Gokhman et al. 2016). So far, paleoepigenetic research has centered on the reconstruction of one type of epigenetic modification, known as cytosine methylation, where a methyl group ($-CH^3$) is added to cytosines in CpG dinucleotide contexts. Cytosine methylation influences chromatin structure and regulates gene expression, and has been implicated in developmental processes, disease etiologies, and mediating interactions between the genome and its physical and social environments (Thayer and Kuzawa 2011, Thayer and Non 2016, Vinkers et al. 2016).

Previous studies have relied on two primary methods to reconstruct ancient methylation patterns: damage-dependent analysis (DDA) and bisulfite sequencing (BS-Seq). Most studies of cytosine methylation in aDNA have used DDA to infer methylation patterns from post-mortem damage to cytosines. This method exploits the fact that methylated and unmethylated cytosines degrade differently after death. Specifically, unmethylated cytosines degrade to uracils, while methylated cytosines degrade to thymines (Figure 2.1). When uracils are removed using aDNA damage repair protocols (Briggs et al. 2010, Gokhman et al. 2014) or through library construction with DNA proofreading enzymes (Pedersen et al. 2014), the remaining thymine misincorporations that stem from

the degradation of methylated cytosines can be used to estimate cytosine methylation. Using patterns of thymine misincorporations, genome-wide methylation patterns have now been inferred in dozens of ancient humans, Neanderthals and Denisovans, and many ancient mammals including horses, polar bears, mammoths, and aurochs (Briggs et al. 2010, Pedersen et al. 2014, Gokhman et al. 2014, Seguin-Orlando 2015, Hanghøj et al. 2016).

Alternatively, bisulfite sequencing (BS-Seq) approaches have been used to reconstruct methylation in aDNA. Because methylated cytosines are resistant to sodium bisulfite treatment ($NaHSO_3$), while unmethylated cytosines are converted to uracils, any cytosines detected after bisulfite conversion are methylated (Figure 2.1). BS-Seq has previously been used to detect cytosine methylation in one ancient bison (Llamas et al. 2012), ancient Egyptian barley (Smith et al. 2014), and ancient humans from a variety of archaeological sites in the Americas (Smith et al. 2015).

Figure 2.1: Detecting methylation statuses of cytosine residues from their deamination products. (adapted from Smith et al. 2015)



5-mc: 5-methylcytosine; 5-hmc: 5-hydroxymethylcytosine; 5-ms: 5-methylenesulfonate; 5-hmu: 5-hydroxymethyluracil; NaHSO3: Sodium bisulfite. (*A*) Unmethylated cytosines are converted to uracil at high efficiency by bisulfite conversion and at low efficiency by post-mortem deamination. After conversion, no methylation is detected by either bisulfite sequencing or damage-dependent analysis. (*B*) Methylated cytosines are unaffected by bisulfite conversion, while post-mortem deamination converts methylated cytosines to thymines. Methylation is detected by the presence of undamaged cytosines in bisulfite sequencing, and by the presence of thymines at damaged positions in misincorporation analysis. (*C*) Hydroxymethylated cytosines are converted to cytosine 5-methelensulfonate by bisulfite conversion, and 5-hydroxymethyluracil by post-mortem deamination. Methylated cytosines are detected at undamaged positions by bisulfite sequencing, but cannot be discriminated from non-hydroxylated methylcytosines using this method. It is currently unclear whether misincorporation analysis will be able to detect methylation in the form of 5-hydroxymethyluracil, but the UDG-endoVIII approach may be able to do so.

There are a variety of tradeoffs entailed with the different methodological approaches for reconstructing methylation patterns in aDNA. BS-Seq provides high resolution methylation data, and can be used to assess the methylation status of single cytosines. This method has successfully been applied to samples that have relatively good preservation of nuclear aDNA, including bison remains preserved in permafrost (Llamas et al. 2012) and human remains up to 6000 years old (Smith et al. 2015). However, because

sodium bisulfite treatment degrades up to 90% of a sample's DNA (Grunau et al. 2001, Tanaka and Okamoto 2007), it is less feasible for samples with poor aDNA preservation. Conversely, DDA can be used to reconstruct methylation patterns without inducing further chemical degradation to aDNA. However, these approaches rely on post-mortem deamination of cytosines, which is a stochastic and incomplete process that is many orders of magnitude less efficient than bisulfite conversion. Because only a fraction of cytosines are converted to uracils by post-mortem processes, methylation levels cannot be estimated at single nucleotide resolution using DDA. Instead, methylation levels must be reconstructed over regions spanning tens or hundreds of nucleotides at a time to maximize the probability that a single cytosine in a given genomic window will be deaminated. This approach therefore yields much lower resolution than BS-Seq. Thus, the feasibility of different methods of reconstructing ancient methylation patterns is driven by considerations such as sample age, rarity, and aDNA preservation, and different methods yield different scales of paleoepigenetic data.

While paleoepigenetics promises new insights into evolutionary processes and lived experiences in ancient societies, such inferences will be constrained by the resolution of paleoepigenetic data. For DDA methods, the interaction between deamination rate and paleoepigenetic resolution has not been well characterized in the literature, nor has there been consideration of how this interaction may affect the kinds of inferences that can be made from paleoepigenetic data. In this study, we characterize the relationship between cytosine deamination rate and epigenetic resolution in aDNA from human skeletal samples, and assess what kinds of evolutionary and life-history inferences could be drawn given various scales of epigenome resolution.

MATERIALS AND METHODS

Bioarchaeological Samples

We obtained dental and skeletal samples of five individuals from different archaeological localities ranging in age from approximately 180-9000 years old (Table 2.1). Each of the dental samples included in this study received approval for genetic analysis. Members of the Kenzaitze tribe contacted Ripan Malhi for genetic analysis of the KEN9 individual. Memoranda of understanding (MOUs) containing approvals for genetic analysis for KEN9 were signed with members of the Kenzaitze Tribe and the Village of Tyonek. Genetic analysis of the PRH532 and PRH940 individuals was done in collaboration with the Metlakatla and Lax Kw'alaams communities, and tribal councils provided approvals for genetic analysis of these individuals. For the PT11 individual, we obtained permission for genetic analysis from the Secretaria de Estado de Cultura, Direcion Provincial de Antropologia in Catamarca, Argentina, with support from the local community in Antofagasta de la Sierra.

Table 2.1: Bioarchaeological Samples.

Sample ID	Burial Location	Age (ybp)
B10	Grassmere Plantation, Nashville, Tennesee	180
KEN9	Kenai Peninsula, Alaska	200
PRH532	Prince Rupert Harbor, British Columbia	2000
PRH940	Lucy Island, British Columbia	5600
PT11	Antofagasta de la Sierra, Argentina	9043

DNA Extraction, Library Construction, and Sequencing

The surfaces of ancient dental and skeletal samples were decontaminated by immersion in 6% sodium hypochlorite (full strength bleach) for 3-10 minutes and then

rinsed 2-3 times with DNA-free water. Samples were then UV irradiated in a 254-nm emitting DNA crosslinker for 5-10 minutes on each side and dried overnight. For samples B10, KEN9, PRH532, and PRH940, 200 mg of powder was obtained from the tooth root using a dremel tool fitted with a dental burr. Ancient DNA was then extracted from tooth powder using the Qiagen MinElute column based method reported in Lindo et al. (2017). For sample PT11, aDNA was obtained using the silica dioxide, guanidinium thiocyanate method reported in Rohland and Hofreiter (2007) but with the modifications reported in Bolnick et al. (2012) for minimally destructive extraction.

DNA libraries were constructed with 5 ng–1 μg of each aDNA extract using the NEBNext Ultra DNA Library Preparation Kit for Illumina (New England Biolabs) following the manufacturer's protocol. Library preparations included incubations with USER enzyme to remove any uracils that had been formed as a result of post-mortem deamination of unmethylated cytosines (Figure 2.1). Samples B10, KEN9, and PRH532 were treated with a partial UDG repair for 15-minutes, similar to the protocol described by Rohland et al. (2014), while PRH940 and PT11 were treated with a full UDG repair for three hours following Briggs et al. (2010). The partial repair protocol is intended to maintain a small fraction of the misincorporations that stem from the deamination of unmethylated cytosines. Because this form of cytosine damage is characteristic of degraded aDNA, but not observed in sequences obtained from contemporary samples, the fraction of misincorporations remaining after partial UDG repair allow for the authentication of aDNA sequences. In contrast, the full repair protocol is designed to remove virtually all the uracils stemming from the degradation of unmethylated cytosines. Following full repair, the remaining fraction of C-to-T misincorporations stem largely from thymines that result from the degradation of methylated cytosines, which are not removed via UDG

repair. Sample-specific DNA barcodes were ligated to each sample library in the aDNA facility, and a portion of the libraries were pooled and submitted for shotgun sequencing on the Illumina HiSeq 2000 platform at the High-Throughput Sequencing Division of the W. M. Keck Biotechnology Center at the University of Illinois Urbana-Champaign. Whole mitogenome sequences were determined from resulting shotgun reads by alignment to the Cambridge Reference Sequence (rCRS). A second portion of each library was enriched for sites of epigenetic regulation using the SureSelect Methyl-Seq Target Enrichment System for Illumina (Agilent Technologies) following the manufacturer's protocol. Libraries were then pooled and submitted for sequencing on the Illumina HiSeq 2000 platform at the High-Throughput Sequencing Division of the W. M. Keck Biotechnology Center at the University of Illinois Urbana-Champaign.

Authentication

Ancient DNA obtained from archaeological remains is highly degraded and requires strict precautions to minimize contamination from exogenous sources of DNA and authenticate results (Kaestle and Horsburgh 2002, Paabo et al. 2004, Gilbert et al. 2005, Willerslev and Cooper 2005, Shapiro 2012). All phases of work with bioarchaeological samples, including aDNA extractions, Illumina library constructions, and library enrichments were conducted in specialized aDNA facilities at the University of Illinois Urbana-Champaign and the University of Texas at Austin. Both aDNA facilities are restricted-access, positive air pressure, HEPA-filter ventilated spaces with overhead UV-irradiating lights that are dedicated to pre-PCR analyses of aDNA. The post-PCR facilities are located in separate buildings, and all movement of materials and personnel was unidirectional (from pre-PCR to post-PCR facility) to prevent contamination from highly concentrated, post-PCR amplified DNA. Additional precautions included the use of sterile

and disposable hooded coveralls, hair covers, face masks, sleeve covers, dedicated shoes, and two pairs of gloves. We frequently decontaminated laboratory benchtops and equipment with 6% sodium hypochlorite (full strength bleach), and decontaminated the entire lab space weekly with a 3% sodium hypochlorite solution (1:1::bleach:water, v/v). The facilities were also irradiated with a 254-nm emitting overhead UV light for 12 hours following each use, while tubes, containers, and reagents were UV irradiated (when possible) in a 254-nm emitting DNA cross-linker for 15 minutes prior to use. Negative controls were included in every phase of sample preparation, including blanks for DNA extraction and Illumina library builds. Each library included sample-specific barcodes so we could computationally exclude any non-endogenous sequences introduced after library construction (i.e., during the subsequent library enrichment and Illumina sequencing steps).

We conducted three sets of *a posteriori* computational analyses to 1) verify that resulting Illumina sequencing reads showed deamination patterns that are characteristic of degraded aDNA, 2) assess the potential impacts of exogenous DNA contamination, and to 3) evaluate whether the dinucleotide contexts of cytosine misincorporation rates match expectations for *in vivo* methylation. First, each sequence dataset was analyzed using MapDamage 2.0 (Jónsson et al. 2013) to assess misincorporation patterns and to evaluate whether the sequence reads show expected patterns of C-to-T transitions concentrated towards fragment ends, which is characteristic of aDNA (Briggs et al. 2010). While UDG-repair does remove many of the misincorporations in DNA libraries, we still expect to see signs of DNA degradation because the use of partial-UDG repair protocols is designed to maintain a small fraction of misincorporations (Rohland et al. 2014). In addition, even when using full repair protocols, some cytosine misincorporations will still remain because 1) UDG treatment cannot remove uracils that stem from the deamination of unmethylated

cytosines if those uracils are not phosphorylated (which can happen at the ends of degraded fragments), and 2) UDG cannot remove the thymines that result from the deamination of methylated cytosines.

Next, we used mitogenome reads to evaluate the rates of non-endogenous contamination from deamination patterns using the program Schmutzi (Renaud et al. 2015). In addition, rates of contamination were estimated from mitogenome sequences by calling an endogenous consensus, and then comparing that consensus sequence to a database of possible contaminants using Schmutzi (Renaud et al. 2015) and contamMix (Fu et al. 2013). Finally, we assessed whether deamination rates were diagnostic of *in vivo* methylation by evaluating the dinucleotide contexts of C-to-T misincorporations. While methylation has been reported in a variety of dinucleotide contexts (CpA, CpC, CpG, and CpT), the vast majority of methylated cytosines in mammals are found in CpG dinucleotides, when cytosines immediately precede a guanine in the DNA sequence (Patil et al. 2014). Because most uracils resulting from the degradation of unmethylated cytosines were removed with UDG treatment, the remaining fraction of C-to-T misincorporations in the DNA sequencing reads should stem from thymines that result from the degradation of methylated cytosines in CpG dinucleotides. We therefore compared the rates of C-to-T misincorporations in each of the four dinucleotide contexts (CpA, CpC, CpG, and CpT) to evaluate whether these misincorporations are diagnostic of methylated cytosines (Briggs et al. 2010, Gokhman et al. 2014, and Pedersen et al. 2014). If deamination reflects *in vivo* methylation patterns, C-to-T transitions should be relatively higher in CpG dinucleotides than in other dinucleotide contexts.

Reconstruction of Cytosine Methylation

As a result of post-mortem processes, a fraction of the unmethylated cytosines in the genome degrade to uracils, while a fraction of the methylated cytosines degrade to thymines (Figure 2.1). When uracils are removed by incubation with UDG, the remaining fraction of C-to-T misincorporations in the genome should result from the deamination of methylated cytosines. Previous studies have shown that rates of C-to-T misincorporations in CpG dinucleotides can be used to reconstruct methylation patterns in aDNA (Briggs et al. 2010, Pedersen et al. 2014, Gokhman et al. 2014, Seguin-Orlando et al. 2015, Hanghøl et al. 2016).

Building on these findings, we developed a computational pipeline to infer methylation patterns from Agilent target-enriched aDNA sequence data. Briefly, sequencing adapters and sample barcodes were removed using the cutadapt package in python. We then removed the first and last nucleotide of every read, because USER-mediated removal of uracils is prohibited whenever terminal uracils are not phosphorylated. Since such uracils would result from the degradation of unmethylated cytosines, they would confound misincorporation-based estimates of cytosine methylation. Reads were further trimmed using fastqc quality filters. Sequencing reads were aligned to the human reference sequence (hg19) using bwa mem and duplicates were removed via samtools rmdup. Using samtools mpileup and bcftools, base composition was determined at every nucleotide position in the genome that was covered by at least one read. Next, we calculated the fraction of thymine misincorporations present in CpG dinucleotides following the approaches reported in Gokhman et al. (2014) and Pedersen et al (2014). To control for the influence of polymorphic sites on C-to-T misincorporation rates, we disregarded any positions where we observed a greater or equal proportion of G-to-A

transitions on the second position of CpG dinucleotides on the negative strands. We also disregarded any C-to-T transitions in known polymorphic positions reported in the NCBI dbSNP database.

Statistical Analysis

Based on previous studies (Pedersen et al. 2014, Gokhman et al. 2014), we defined a summary statistic, M_{res}, that can be used to determine the optimal methylome resolution for each aDNA sample. M_{res} is determined by calculating the minimum number of consecutive nucleotides (window size) necessary to produce the maximum correlation between cytosine deamination patterns in aDNA and methylation levels of corresponding genomic regions in publicly available Reduced Representation Bisulfite Sequencing (RRBS) data generated from contemporary osteoblasts (ENCODE: ENCSR000DEU). As of this study, no epigenomic data from contemporary dental samples was available. However, my analysis of LINE-1 methylation patterns in Chapter Three of this dissertation indicate that, at least for these retrotransposable loci, methylation patterns are highly correlated between several different tissues, and teeth and bone are expected to show highly similar patterns of cytosine methylation.

In this study, M_{res} was determined for each autosomal chromosome across six different window scales, ranging from 50-300 nucleotide positions (nps) (50, 100, 150, 200, 250, and 300). X and Y chromosomes were excluded from the analysis. Next, we assessed how methylome resolution relates to aDNA preservation using linear regression analysis of M_{res} against global genomic deamination rates for each genome from MapDamage 2.0 analyses. Finally, we assessed whether C-to-T misincorporations were greater in CpG dinucleotides than other dinucleotide contexts (CpA, CpC, and CpT) using the Kruskal-Wallis H Test. Shapiro-Wilk tests, quantile-quantile plots, and histogram plots

indicated that the misincorporation data were not normally distributed, so a non-parametric comparison of variance was used. Statistical analyses were performed using custom pipelines in the python, [R], and bash environments, and plots were generated using the ggplot2 package in [R] (R Core Team 2017).

RESULTS

Shotgun Sequencing and Mitogenome Analysis

Mitogenome analysis for samples KEN9, PRH532, PRH940, and PT11 yielded mtDNA haplogroups that are commonly found among Native Americans (Table 2.2). The mitogenome analysis of sample B10 yielded a mtDNA haplogroup that is common throughout Africa and the African diaspora (Table 2.2). These haplogroup designations are consistent with expectations based on the archaeological context of each site, they are distinct from those of lab members involved in this project, and are unlikely to stem from contamination.

Table 2.2: Results of Mitogenome Analysis.

Sample	mtDNA Haplogroup	mtDNA Coverage
B10	L1c2b1c	5.8X
KEN9	A2	19.1X
PRH532	A2p	20.0X
PRH940	A2ag	51.6X
PT11	D4h3a5	19.1X

Schmutzi analyses of contamination rates using mitogenome reads yielded variable results. While the PRH940 and PT11 samples were estimated to have low rates of non-endogenous sequences present, KEN9 and PRH532 yielded higher estimates of contamination (Table 2.3). Sample B10 yielded too few reads and was excluded from this analysis. However, these estimates stem solely from deamination patterns, and because the samples lack high levels of deamination because of preservation and/or UDG treatment, all samples in this analysis fell below the deamination levels that are recommended for Schmutzi analysis (5% deamination). Sequencing depth for each sample was also below the recommended minimum for Schmutzi analysis (>500 million molecules). Therefore,

the confidence in contamination estimates from this approach is limited. Contamination rates were also estimated via Schmutzi and contamMix analysis by determining a consensus for the endogenous mitochondrial sequence and then comparing any non-endogenous sequences to a database of potential mtDNA contaminants.

Table 2.3: Estimates of non-endogenous contamination from patterns of DNA deamination.

Sample	Bayesian Estimates of Percent Contamination (ranging from 0-1)		
	Lower	**Average**	**Upper**
KEN9	0.45	0.42	0.48
PRH532	0.99	0.98	0.99
PRH940	0	0	0.01
PT11	0	0	0.95

Nuclear DNA Target Capture and Reconstruction of Cytosine Methylation

Following Agilent Methyl-Seq target capture, each of the five samples yielded nuclear DNA sequences enriched for regions of the genome that are known to be sites of cytosine methylation (Table 2.5). However, sample B10 did not yield sufficient genome coverage and was excluded from further analysis. While coverage of the remaining genomes is also relatively low, it was sufficient enough to investigate how methylome resolution is related to deamination rates in the regions targeted by the MethylSeq probes. MapDamage analysis of nuclear DNA for PRH532, PRH940, and PT11 shows that C-to-T misincorporations are concentrated near the 5' ends of sequencing reads, a pattern that is considered to be diagnostic of aDNA (Rohland et al. 2014, Figure 2.2). MapDamage results also show that genomic rates of deamination vary between the four targeted genomes in this study (Table 2.6). Because each of the DNA libraries were treated with UDG to remove uracils that result from the deamination of unmethylated cytosines, the remaining fraction

of C-to-T misincorporations for each of these genomes is low and stems largely from the deamination of methylated cytosines.

Table 2.4: Results of Agilent MethylSeq Capture.

Sample ID	Coverage	Total Reads	Mapped Reads
B10	1.1x	86,086,966	50,376
KEN9	1.3x	136,745,581	126,194
PRH532	1.3x	12,709,428	1,182,365
PRH940	1.5x	55,396,576	126,298
PT11	1.3x	24,399,975	9,584,519

Figure 2.2: MapDamage analysis showing frequencies and distributions of C-to-T misincorporations.

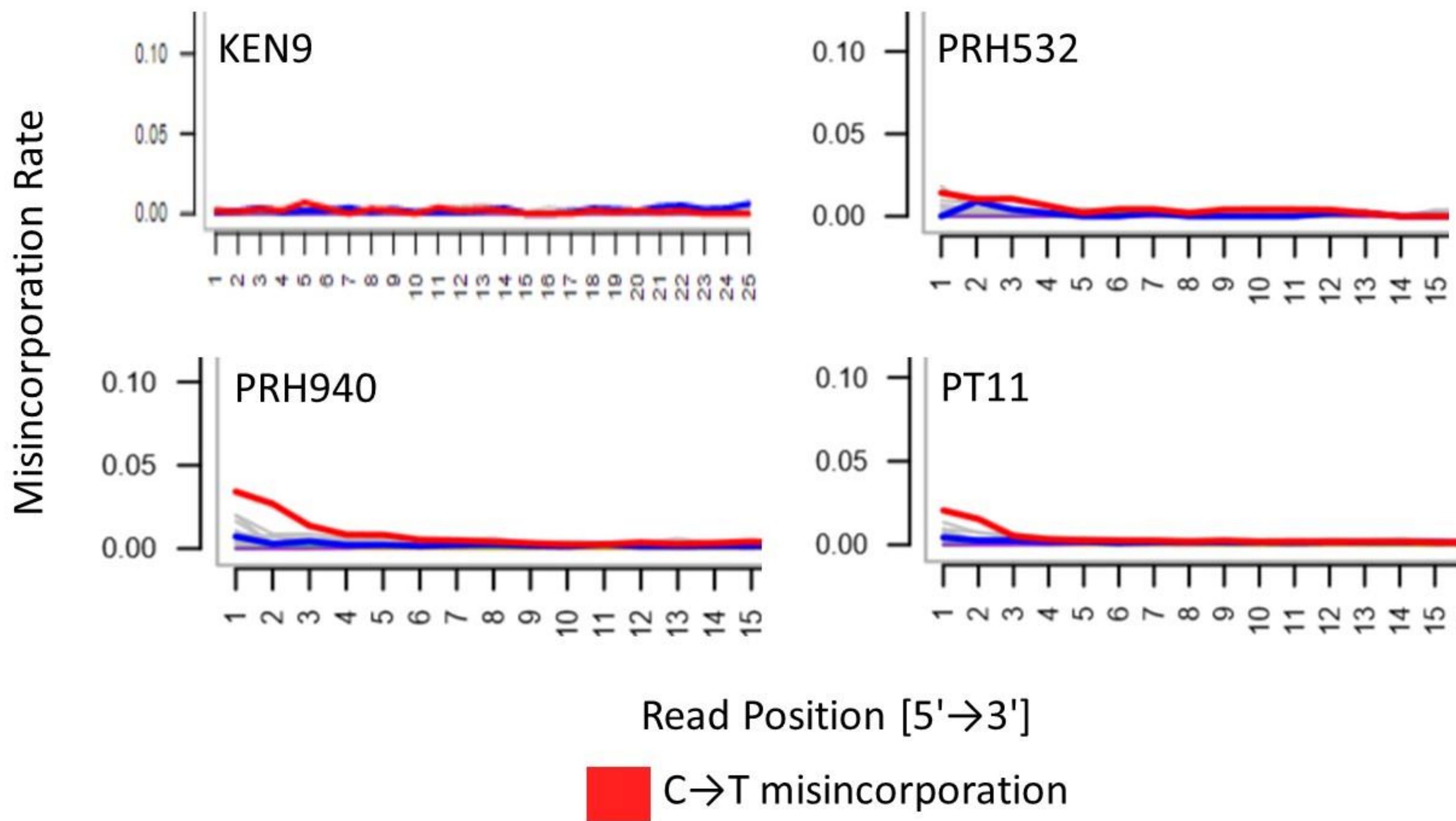
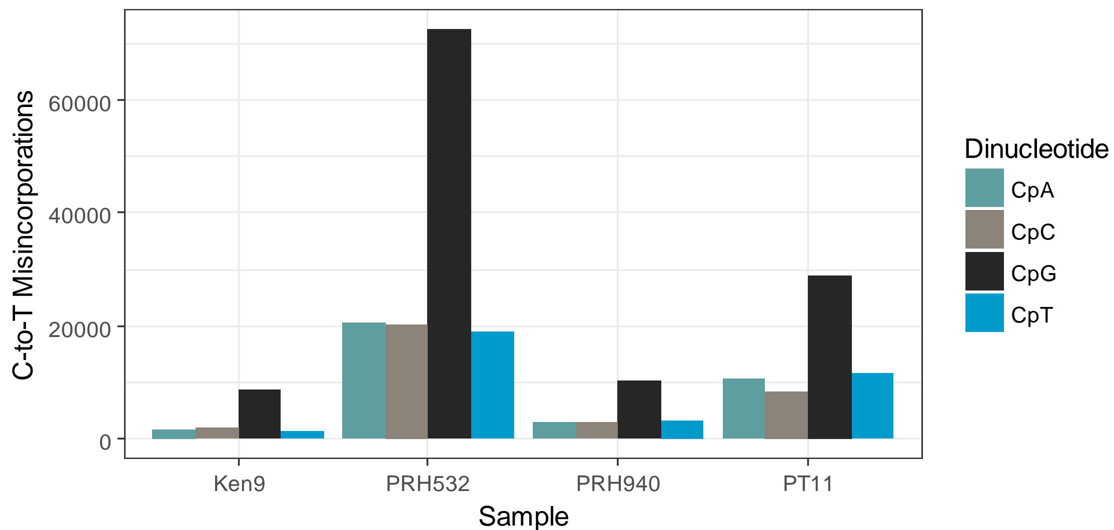


Table 2.5: Rates of genomic deamination for four Agilent-targeted genomes.

Sample	Genomic Deamination Rate
KEN9	0.055
PRH532	0.063
PRH940	0.112
PT11	0.059

When we analyzed cytosine misincorporation rates by their dinucleotide context (Figure 2.3), we found that C-to-T transitions were highest in CpG dinucleotides, and were much lower in the other three dinucleotide contexts (CpA, CpC, and CpT). While Kruskal-Wallis H Tests indicated that these differences were not statistically significant (p=0.40), this finding nonetheless suggests that the majority of C-to-T transitions stem from the deamination of methylated cytosines (Figure 2.1), and that these misincorporations can be used to estimate *in vivo* methylation levels.

Figure 2.3: C-to-T misincorporations rates by dinucleotide context.



Finally, methylome resolutions were estimated from patterns of C-to-T misincorporations in CpG dinucleotides using the Agilent-targeted nuclear aDNA data. While sample B10 was excluded from analysis due to insufficient read density, M_{res} was evaluated for samples KEN9, PRH532, PRH940, and PT11. We found that methylome resolutions varied substantially across autosomes and that the chromosomes that yielded the highest resolutions were different between samples. The probability that any cytosine will become deaminated has been shown to be uniform across the genome (Gokhman et al. 2014). However, while there is an equal likelihood of deamination across all chromosomes, our results indicate that chromosomal deamination rates are uneven, with some chromosomes showing higher rates of deamination than others (Figure 2.4). When methylome resolutions were estimated across all autosomes for each sample, values ranged between window sizes of 50-300 nps (Figures 2.4 and 2.5). For samples KEN9, PRH940, and PT11, M_{res} values of 50 nps generated the highest correlations with methylation patterns in contemporary osteoblasts, and larger window sizes generated progressively negative correlations for each of these samples. For each of these genomes, then, the smaller window sizes tested seem to have been slightly more predicative of *in vivo* methylation patterns than the larger ones. However, even at a window size of 50 nps, the correlations with endogenous methylation patterns were uniformly weak and sometimes negative. In other words, even the best window sizes identified in our analyses were neither strong nor precise predictors of *in vivo* methylation levels. Sample PRH532 generated stronger correlations with *in vivo* methylation patterns in contemporary osteoblasts, and M_{res} steadily increased up to the maximum window size of 300 nps. In this sample, then, the larger the window size, the better the correlation with *in vivo* methylation. Combining MapDamage and M_{res} results into a linear regression analysis, we found that genomic

deamination levels and methylome resolutions are inversely related (R^2= -0.62), with higher deamination rates yielding more fine-scale methylome resolutions. However, this effect was not statistically significant (p=0.71).

Figure 2.4: Correlation of C-to-T misincorporation rates with *in vivo* methylation of osteoblasts.

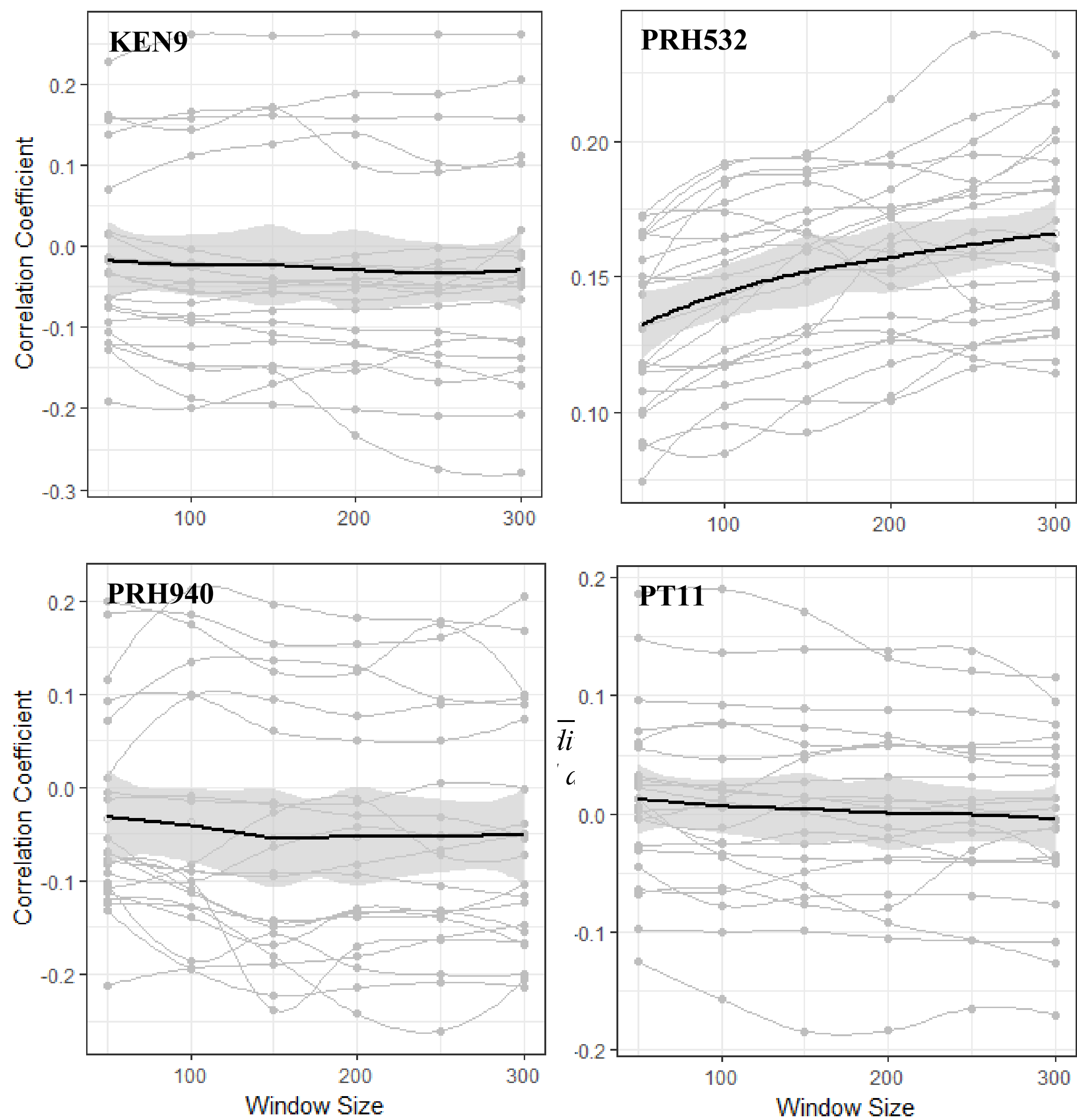
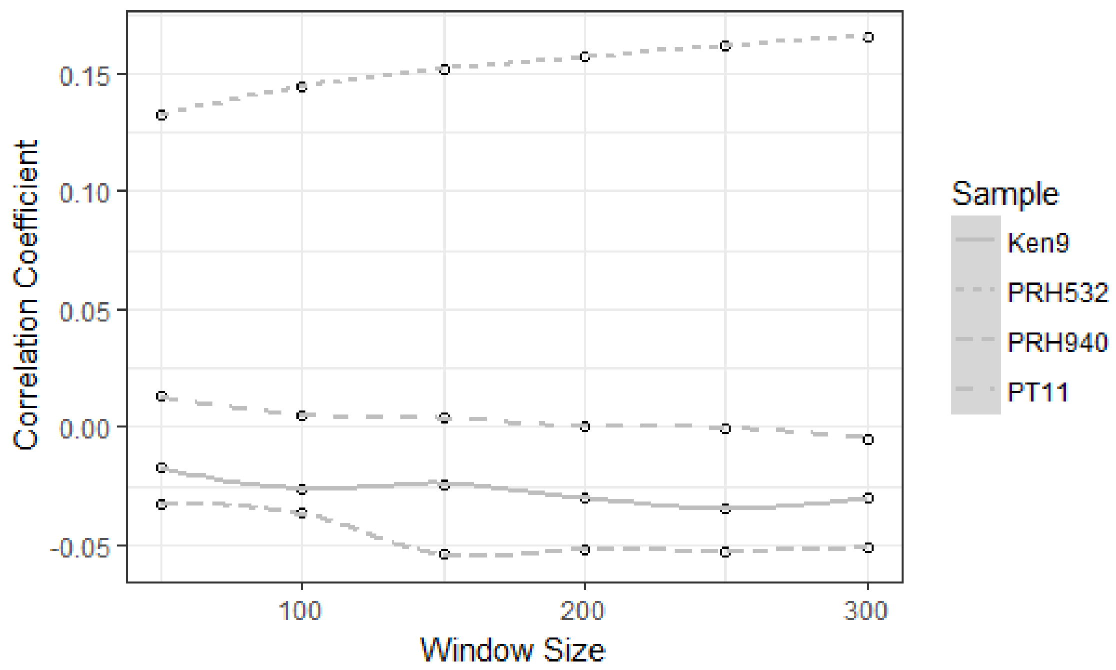


Figure 2.5: Fitted M_{res} values for the four ancient targeted genomes.



DISCUSSION

The results presented in this study are likely to be derived from endogenous aDNA. While deamination rates for each of the genomes are low following UDG-repair, MapDamage results showed that the remaining C-to-T transitions are concentrated at the 5' end of reads, which is characteristic of degraded aDNA. In addition, the mitogenome consensus sequences for each sample fit with expectations based on their archaeological contexts, and do not correspond to any mitogenome sequences of lab members involved in the project, suggesting that samples were not contaminated during laboratory processing. However, Schmutzi estimates of exogenous contamination based on mitogenome reads yielded variable results. While estimates of exogenous contaminants for PRH940 and PT11 were very low, KEN9 and PRH532 may have higher rates of exogenous contamination. However, these contamination estimates should be interpreted with caution for two reasons. First, all four samples had very low rates of deamination following UDG repair, which can lead to overestimations of endogenous contamination when these estimates are based on deamination patterns alone. Second, when using the Schmutzi implementation (Renaud et al. 2015), there were not enough molecules present to estimate contaminants by calculating the fraction of reads that do not match the expected consensus sequence. To evaluate whether the results from Schmutzi analysis were reliable, we calculated the fraction of reads that do not align to the predicted consensus sequences using contamMix (Fu et al. 2013), which does require high depth of coverage for estimating contamination rates. Finally, our analysis of thymine misincorporations shows that C-to-T transitions are vastly (though not significantly) over-represented in CpG dinucleotide contexts relative to other dinucleotide contexts, suggesting that these thymines stem from the deamination of methylated cytosines which are not removed via UDG-repair.

Because stable epigenetic differences can accompany morphological and other phenotypic changes, researchers have suggested that reconstructing epigenetic marks from aDNA could inform our understanding of past evolutionary processes, including speciation, animal and plant domestication, and other morphological changes that followed events like the Neolithic revolution (Orlando and Willerslev 2014, Gokhman et al. 2016). In addition, because changes in cytosine methylation can be more plastic and responsive to differences in lived experiences, including nutrition, stress, and other factors, it may also be possible to use ancient epigenetic marks to understand non-evolutionary processes and provide new insights into past lifeways. However, different environmental and evolutionary factors likely shape the epigenome at different scales, ranging from a single cytosine to many thousands of cytosines. For example, factors such as diet and stress can produce epigenetic changes at multiple different scales. Dietary and stress differences can influence methylation at the global genomic level, through changes in the methylation of retrotransposable elements (Zhang et al. 2011a, Zhang et al. 2011b). However, because methylation of repetitive elements can be shaped by the interaction of many factors simultaneously, these measures of global genomic methylation lack a single biological or functionally specific meaning. In contrast, dietary and stress differences may also be associated with highly localized, functionally specific epigenetic changes. For example, exposure to stress can alter NR3C1 methylation in as few as 1-5 cytosines in exon $1_F/1_7$ (Vinkers et al. 2016). Therefore, the resolution with which paleoepigenomic studies can reconstruct methylation patterns in ancient genomes will greatly influence the kinds of inferences that can be made from ancient methylomes.

Because DDA-based inferences of methylation rely on the stochastic and incomplete conversion of cytosines by post-mortem deamination, they can only be used to

estimate methylation at low resolutions over many dozens or even hundreds (or thousands) of nucleotides. In our analysis, M_{res} ranged from 50-300 nps given deamination rates of 0.06-1%. Such resolutions will make it possible to infer larger-scale epigenetic effects, such as the methylation states of repetitive elements or differences in promoter/gene body methylation that could provide rough measures of gene activity. However, these resolutions are not well suited to more fine-scale epigenetic analyses, such as determining the methylation states of transcription factor binding sites in the promoter regions of dietary or stress-related genes, which often entails determining the methylation state of a single specific cytosine. Thus, while these DDA methods can be used to give a general assessment of larger-scale methylation patterns, they lack the resolution required to detect highly localized and functionally specific epigenetic changes.

Our results point to important tradeoffs between the two main approaches that have been used to reconstruct cytosine methylation in aDNA. For samples with highly degraded aDNA and high rates of cytosine deamination, DDA methods are well suited for producing regional estimates of methylation that can be used to infer larger scale changes in genomic methylation. These methods may provide the only practical way to reconstruct methylation in highly degraded or rare samples where the destructive effects of bisulfite treatment would be infeasible, but they will likely always limit the kinds of inferences that can be obtained. Our findings suggest that it is unlikely that DDA methods could provide insights into functionally meaningful, site-specific epigenetic changes that accompany events like ancient famines or warfare. In contrast, BS-seq can be used to produce high resolution methylation data, but it will only be possible with samples with sufficiently well-preserved nuclear DNA because bisulfite treatment greatly reduces the number of template molecules available for analysis (Llamas et al. 2012, Smith et al. 2014, Smith et al. 2015). It should

be noted that BS-Seq is currently the *only* method that has been successfully applied to aDNA that can produce the methylome resolutions necessary to evaluate the fine-scale, functionally meaningful effects of ancient lived experiences. To date, BS-Seq is the only current method that could be used to reconstruct methylation in younger or more well-preserved samples which lack the degree of cytosine deamination that is required for DDA methods. Future studies that wish to reconstruct the effects of ancient lived experiences, including the functionally specific outcomes of dietary differences and experiences of traumatic violence, must bear such distinctions in mind. In addition, future research could investigate whether other high-resolution methods of detecting cytosine methylation, such as mass spectrometry (Lin et al. 2016), could be used to detect methylation in degraded aDNA.

The emerging field of paleoepigenetics faces a number of additional limitations and challenges that must be carefully considered in future studies. First, research in epigenetics already faces scrutiny, and has often been the locus of controversy because epigenetic findings have sometimes been seen as contradictory to existing paradigms of evolutionary thought. One example of such controversies can be seen in the ongoing debate around whether epigenetic marks can be in inherited across generations, which has often been seen as contradictory to 1) some of the early definitions of epigenetics that did not include the possibility of meiotic transfer of epigenetic information, and 2) Darwin's theory of evolution by natural selection, which does not explicitly include the possibility that ancestors' experiences might impact the phenotypes of their offspring (Tollefsbol 2014).

Second, because paleoepigenetics involves epigenetic analysis of aDNA, this subfield of epigenetic research faces even more scrutiny than epigenetic studies of living organisms. Any research with aDNA faces a number of laboratory and analytical

challenges due to the post-mortem degradation of DNA sequences (Kaestle and Horsburgh 2002, Paabo et al. 2004, Gilbert et al. 2005, Willerslev and Cooper 2005, Shapiro 2012). Ancient DNA is highly fragmented, plagued by sequence damage, and highly prone to exogenous contamination. Because of these issues, some of the earliest findings in the field of aDNA were shown to be erroneous. This led to the development of stringent criteria for authenticating aDNA results (Cooper and Poinar 2000). While the specific criteria for authenticating aDNA results have greatly changed following the advent of next generation sequencing technologies, they remain a central concern in the field, and are likely to be of even greater concern for studies of epigenetic marks.

As we have demonstrated, patterns of DNA degradation impose limits on the scale of epigenetic changes that can be inferred with confidence. Given these challenges, it is important that emerging research in paleoepigenetics does not overstate its potential for reconstructing ancient lived experiences. While DDA methods have been proposed as one method for investigating ancient lived experiences, our research suggests that these insights have important constraints that limit what can be inferred about ancient lifeways.

REFERENCES

Bolnick DA, et al. 2012. Nondestructive sampling of human skeletal remains yields ancient nuclear and mitochondrial DNA. Am J Phys Anthropol 147:293–300.

Briggs AW, et al. 2010. Removal of deaminated cytosines and detection of in vivo methylation in ancient DNA. Nucleic Acids Res 38:1–12.

Cooper A and Poinar HN. 2000. Ancient DNA: do it right or not at all. Science 289:1139.

Fu Q, et al. 2013. A revised timescale for human evolution based on ancient mitochondrial genomes. Curr Biol 23:553–559.

Gilbert MTP, et al. 2005. Assessing ancient DNA studies. Trends Ecol Evol 20:541–4.

Gokhman D, et al. 2016. Epigenetics: It's Getting Old. Past Meets Future in Paleoepigenetics. Trends Ecol Evol 31:290–300.

Gokhman D, et al. 2014. Reconstructing the DNA Methylation Maps of the Neandertal and the Denisovan. Science (80) 344:523–528.

Grunau C, et al. 2001. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. Nucleic Acids Res 29:E65–5.

Hanghøj K, et al. 2016. Fast, accurate and automatic ancient nucleosome and methylation maps with epiPALEOMIX. Mol Biol Evol.

Jónsson H, et al. 2013. MapDamage2.0: Fast approximate Bayesian estimates of ancient DNA damage parameters. Bioinformatics 29:1682–1684.

Kaestle F and Horsburgh KA. 2002. Ancient DNA in anthropology: Methods, applications, and ethics. Am J Phys Anthropol 119:92–130.

Lin X, et al. 2016. Mass Spectrometry Based Ultrasensitive DNA Methylation Profiling Using Target Fragmentation Assay. Anal Chem 88(2): 1083–1087.

Lindo AJ, et al. 2016. Demographic and immune-based selection shifts before and after European contact inferred from 50 ancient and modern exomes from the Northwest Coast of North America. bioRxiv:1–16.

Llamas B, et al. 2012. High-resolution analysis of cytosine methylation in ancient DNA. PLoS One 7:e30226.

Orlando L, Willerslev E. 2014. An epigenetic window into the past? Science (80)345:511–512.

Paabo S, et al. 2004. Genetic Analyses from Ancient DNA. Annu Rev Genet 38:645–679.

Patil V, et al. 2014. The evidence for functional non-CpG methylation in mammalian cells. Epigenetics 9:823–828.

Pedersen JS, et al. 2014. Genome-wide nucleosome map and cytosine methylation levels of an ancient human genome. Genome Res 24:454–466.

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Renaud G, et al. 2015. Schmutzi: estimation of contamination and endogenous mitochondrial consensus calling for ancient DNA. Genome Biol 16:1–18.

Rohland N, et al. 2014. Partial UDG-treatment for screening of ancient DNA. Philos Trans R Soc Biol:1–38.

Rohland N and Hofreiter M. 2007. Ancient DNA extraction from bones and teeth. Nat Protoc 2:1756–1762.

Seguin-Orlando A, et al. 2015. Pros and cons of methylation-based enrichment methods for ancient DNA. Sci Rep 5:11826.

Shapiro B and Hofreiter M (Eds). 2012. Ancient DNA. Methods and Protocols. Methods in Molecular Biology vol 840. Springer, New York.

Smith O, et al. 2014. Genomic methylation patterns in archaeological barley show de-methylation as a time-dependent diagenetic process. Sci Rep 4:5559.

Smith RWA, et al. 2015. Detection of cytosine methylation in ancient DNA from five native American populations using bisulfite sequencing. PLoS One 10:1–23.

Tanaka K and Okamoto A. 2007. Degradation of DNA by bisulfite treatment. Bioorganic Med Chem Lett 17:1912–1915.

Thayer ZM and Kuzawa CW. 2011. Biological memories of past environments: Epigenetic pathways to health disparities. Epigenetics 6:798–803.

Thayer ZM and Non AL. 2015. Anthropology Meets Epigenetics: Current and Future Directions. Am Anthropol 117:722–735.

Tollefsbol T. 2014. Transgenerational Epigenetics. Cambridge: Academic Press.

Vinkers CH, et al. 2015. Traumatic stress and human DNA methylation: a critical review. Epigenomics 7:593–608.

Willerslev E and Cooper A. 2005. Ancient DNA. Proc R Soc B Biol Sci 272:3–16.

Zhang FF, et al. 2011a. Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. Epigenetics 6:623–629.

Zhang FF, et al. 2011b. Dietary Patterns Are Associated with Levels of Global Genomic DNA Methylation in a Cancer-Free Population. J Nutr:1165–1171.

CHAPTER THREE: LINE-1 Methylation in Ancient DNA from Wari and post-Wari Populations in Peru.

BACKGROUND

While the field of paleoepigenetics is breaking new ground with regard to the kinds of information that can be obtained from degraded ancient DNA (aDNA), the focus of the field has been largely methodological thus far. To date, no study has evaluated whether ancient methylation patterns reflect sociopolitical and environmental differences in ancient societies. In this study, we applied recent developments in the field of paleoepigenetics to answer archaeologically-informed questions about the decline of an ancient society of the central Peruvian Andes known as the Wari. In doing so, we provide new insights into how epigenetic data could reflect sociopolitical and environmental change over many centuries of Andean history.

Paleoepigenetics

Paleoepigenetics is an emerging area of aDNA research that reconstructs chemical modifications to DNA obtained from ancient organisms and evaluates their environmental and evolutionary significance. To date, paleoepigenetic research has largely centered on analyzing patterns of one kind of epigenetic modification, known as cytosine methylation, where a methyl group ($-CH^3$) is attached to cytosines in cytosine-guanine (CpG) dinucleotides in the DNA sequence. Cytosine methylation has been extensively studied in living humans and other organisms, where it has been linked with developmental processes, disease etiologies, and mediating interactions between the genome and its physical and social environments (Portella and Esteller 2010, Thayer and Kuzawa 2011, Feil and Fraga 2012, Cantone and Fisher 2013, Thayer and Non 2016). Because stable

epigenetic differences may accompany morphological changes, researchers have suggested that reconstructing epigenetic marks from aDNA can inform past evolutionary processes, including species divergence, animal and plant domestication, and other morphological changes that followed events like the Neolithic revolution (Orlando and Willerslev 2014, Gokhman et al. 2016). In addition, because more plastic changes in cytosine methylation can be shaped by lived experiences, including nutrition, stress, and other factors (Thayer and Kuzawa 2011), it may also be possible to use ancient epigenetic marks to understand non-evolutionary processes, such as the impacts of social inequalities, sociopolitical transitions, and famines that occurred in distant past.

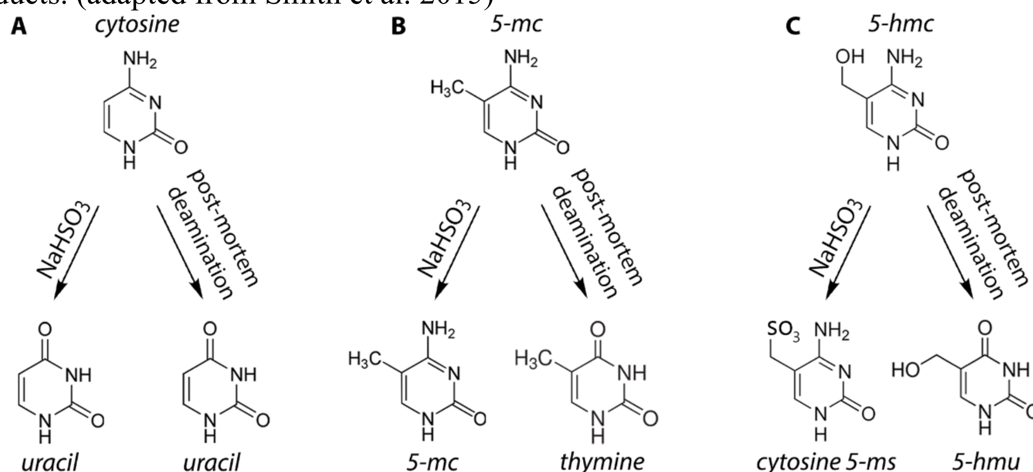
Relatively little research has focused on analyzing epigenetic marks in ancient organisms, but a series of recent studies have shown that cytosine methylation can be reconstructed from aDNA using two methodological approaches: damage-dependent analysis (DDA) and bisulfite sequencing (BS-Seq). Most studies of cytosine methylation in aDNA have used DDA approaches to infer methylation patterns from post-mortem damage to cytosines. This method exploits the fact that methylated and unmethylated cytosines degrade differently after death. Specifically, unmethylated cytosines degrade to uracils, while methylated cytosines degrade to thymines (Figure 3.1). When uracils are removed using aDNA repair protocols (Briggs et al. 2010, Gokhman et al. 2014) or through library construction with DNA proofreading enzymes (Pedersen et al. 2014), the remaining thymine misincorporations that stem from the degradation of methylated cytosines can be used to estimate cytosine methylation. Using patterns of thymine misincorporations, genome-wide methylation patterns have now been inferred in dozens of ancient humans, Neanderthals and Denisovans, and many ancient mammals including horses, polar bears, mammoths, and aurochs (Briggs et al. 2010, Pedersen et al. 2014, Gokhman et al. 2014,

Seguin-Orlando 2015, Hanghøj et al. 2016). Alternatively, bisulfite sequencing (BS-Seq) approaches have been used to reconstruct methylation in aDNA. Because methylated cytosines are resistant to sodium bisulfite treatment ($NaHSO_3$) while unmethylated cytosines are converted to uracils, any cytosines detected after bisulfite conversion are methylated (Figure 3.1). BS-Seq has previously been used to detect cytosine methylation in one ancient bison (Llamas et al. 2012), ancient Egyptian barley (Smith et al. 2014), and ancient humans from a variety of archaeological sites across North America (Smith et al. 2015).

Given limitations in the abundance and availability of archaeological remains for genetic research, and variation in the quality of DNA preservation, there are tradeoffs between DDA and BS-Seq approaches for reconstructing methylation from aDNA. Because DDA methods rely on the presence of deaminated cytosines to detect methylation and do not involve further chemical treatment, this method may be preferable for older or rarer samples where less destructive methodologies may be necessary. DDA methods may also be preferable for highly degraded samples where there is sufficient deamination present to estimate methylation patterns (Smith et al. 2015, Smith et al. 2016, see chapter 2). However, inferring methylation from deamination is less applicable to well-preserved samples where there may not be enough deamination present to reconstruct methylation patterns. Because sodium bisulfite treatment has been shown to degrade up to 90% of input DNA (Grunau et al. 2001, Tanaka and Okamoto 2007), these methods would be infeasible for highly degraded or rarer samples where minimally destructive approaches may be preferred. But BS-seq has proved effective for reconstructing methylation patterns in ancient samples with well-preserved DNA (Llamas et al. 2012, Smith et al. 2015). Finally, there are differences in the overall resolution of methylation that one can achieve with

different methods. While BS-Seq can be used to reconstruct the methylation status of single cytosines (highest resolution), DDA methods provide only regional estimates of methylation over many tens or hundreds of nucleotides (low resolution).

Figure 3.1: Detecting methylation states of cytosine residues from their deamination products. (adapted from Smith et al. 2015)



5-mc: 5-methylcytosine; 5-hmc: 5-hydroxymethylcytosine; 5-ms: 5-methylenesulfonate; 5-hmu: 5-hydroxymethyluracil; NaHSO3: Sodium bisulfite. (A) Unmethylated cytosines are converted to uracil at high efficiency by bisulfite conversion and at low efficiency by post-mortem deamination. After conversion, no methylation is detected by either bisulfite sequencing or misincorporation analysis. (B) Methylated cytosines are unaffected by bisulfite conversion, while post-mortem deamination converts methylated cytosines to thymines. Methylation is detected by the presence of undamaged cytosines in bisulfite sequencing, and by the presence of thymines at damaged positions in misincorporation analysis. (C) Hydroxymethylated cytosines are converted to cytosine 5-methelensulfonate by bisulfite conversion, and 5-hydroxymethyluracil by post-mortem deamination. Methylated cytosines are detected at undamaged positions by bisulfite sequencing, but cannot be discriminated from non-hydroxylated methylcytosines using this method. It is currently unclear whether misincorporation analysis will be able to detect methylation in the form of 5-hydroxymethyluracil, but the UDG-endoVIII approach may be able to do so.

Tissue-Dependent Methylation

Another concern about paleoepigenetic research stems from the fact that methylation patterns often vary from tissue to tissue, reflecting functional differences in gene expression (Christensen et al. 2009, Varley et al. 2013, Lokk et al. 2014). Thus, one potential limitation in epigenetic studies of gene-environment interactions in ancient humans is the lack of aDNA from the most physiologically relevant tissues, which are rarely preserved in archaeological contexts (Campos et al. 2012). For example, for research that is focused on the epigenetic correlates of stress, tissues from the hypothalamic-pituitary-adrenal (HPA) axis would be the most physiologically relevant tissues. However, these soft tissues are only very rarely preserved in archaeological contexts and are therefore largely unavailable for paleoepigenomic research. It is important to note, though, that limitations posed by tissue-dependent methylation also apply to studies of cytosine methylation in living humans, where peripheral blood or saliva are often used in lieu of more invasive tissue sampling. Therefore, physiologically relevant tissues are not always strictly required for epigenetic research, because methylation patterns in peripheral tissues may be correlated with those of physiologically relevant tissues, or show similar responses to the stimulus under investigation.

While a discrete set of methylated sites do differ between tissues, most of the genome shares common DNA methylation patterns across cell types. Recent studies have found that as many as 80% of all methylated regions are expected to be similar across tissues (Ziller et al. 2013). The more developmentally and functionally related tissues are, the more likely it is that their methylation patterns will be correlated. In addition, genes that are functionally relevant to a broad spectrum of different tissue activities, such as those related to stress, immunity, growth, and energy metabolism, would also be expected to

show similar methylation patterns across tissues. Conversely, methylation of genes whose function is highly specialized may be expected to differ between tissues. For most methylated regions, however, patterns observed in teeth and bone are likely to be conserved across tissues. Therefore, while methylation in osseous tissues would not be expected to be functionally related to every environmental stimulus, it could nevertheless serve as a useful biomarker for exposure to certain social and physical environments in the same way that peripheral tissues such as blood and buccal cells have been used in contemporary populations.

It is relatively unclear how patterns of methylation derived from archaeologically available tissues such as teeth, hair, and bone compare with tissues that are more commonly studied in living populations, such as buccal cells or blood. To address some of these issues, we conducted a preliminary analysis to evaluate whether methylation patterns of Long Interspersed Nuclear Elements (LINE-1) are correlated between molars, buccal cells, whole blood, hair, and nails that were obtained from living humans, providing a means to help interpret ancient methylation patterns reconstructed from ancient teeth. LINEs are highly repetitive and CpG-dense sequences that have been widely used as a measure of global genomic methylation (Ricceri et al. 2014). While the full biological meaning and functional significance of LINE-1 methylation remains unclear, differences in LINE-1 methylation have been linked with social and environmental differences including dietary disparities and social inequalities (Zhang et al. 2011a, Zhang et al. 2011b). For example, these studies have indicated that differences in lived experiences that are shaped by gender and ethnic inequalities can lead to a 2-3% decrease in average genomic methylation. We hypothesized that LINE-1 methylation should be correlated between different tissues, as it is not known to be functionally specific to any differentiated cell functions. However,

because LINE-1 methylation is known to be responsive to physical and social environments, we also hypothesized that differences in lived experiences could alter methylation of LINE-1 differently across tissues. In this study, we therefore conducted an analysis of LINE-1 methylation across multiple tissues in individuals that had different life histories. We use these data to interpret LINE-1 methylation patterns reconstructed from ancient skeletal remains.

Overview of Wari Society in the Central Peruvian Andes

Beginning around 600 CE, the Wari emerged as the first expansive state societies in South America (Isbell 2004). From its large urban capitol of Huari in the Ayacucho Basin, the Wari culture spread through much of what is now northern, southern, and coastal Peru, achieving unprecedented influence across a vast area of the central Peruvian Andes (Schreiber 1992). The Wari expansion helped to reshape the sociopolitical organization and material culture of the region (Figure 3.2 and 3.3, Tung 2012). This process is evident in the use of Wari-style architecture across the central Andes, including "patios surrounded by narrow rectangular buildings, niched halls, and D-shaped buildings used for rituals", the transformation of landscapes to increase agricultural production, and the construction of extensive roadways and trade networks (Isbell 1991, Schreiber 1991, Williams 2002, Tung 2012). The influence of Wari culture is also evident in shifting mortuary traditions, in the Nasca region for example, people began interring people together in sepultures with Wari-style grave offerings, a shift from previous mortuary practices where people had more commonly been interred in individual graves (Tung 2012). Other influences on material culture include widespread integration of Wari iconography into ceramic, textile, and metallurgical production throughout many regions of the central and southern Peruvian Andes (Tung 2012). Recent excavations are continuing to shed light on Wari material

culture. Beginning in 2010, a team of archaeologists led by Milosz Giersz and Roberto Pimentel Nita excavated a tomb at the site of El Castillo de Huarmey in northern, coastal Peru. This tomb is one of the only known unlooted tombs from the Wari era, and included the remains of three elite women who were interred with elaborate grave offerings including gold earrings and weaving implements. These findings highlight the extent of Wari influence across northern Peru, which had spread far from urban centers in the Wari heartland. The period from 600-1000 CE, which includes the emergence and expansion of Wari culture, is referred to as the Middle Horizon (MH).

Within the social orders that the Wari imposed, differences in lived experiences, including the quality of people's diet and their risk of exposure to traumatic violence were structured by age, status, geographic, and gender. During Wari times, the marginalization and destruction of certain bodies often served as a way to perform and maintain state power (Tung 2012). Therefore, vulnerability during Wari times was not randomly structured in Wari populations but was intensively shaped by aspects of Wari social life.

Figure 3.2: Geographic locations of Wari archaeological sites in the central Peruvian Andes.

Image adapted from Tung 2012

Figure 3.3: Detail of Wari archaeological sites in the Ayacucho Basin.

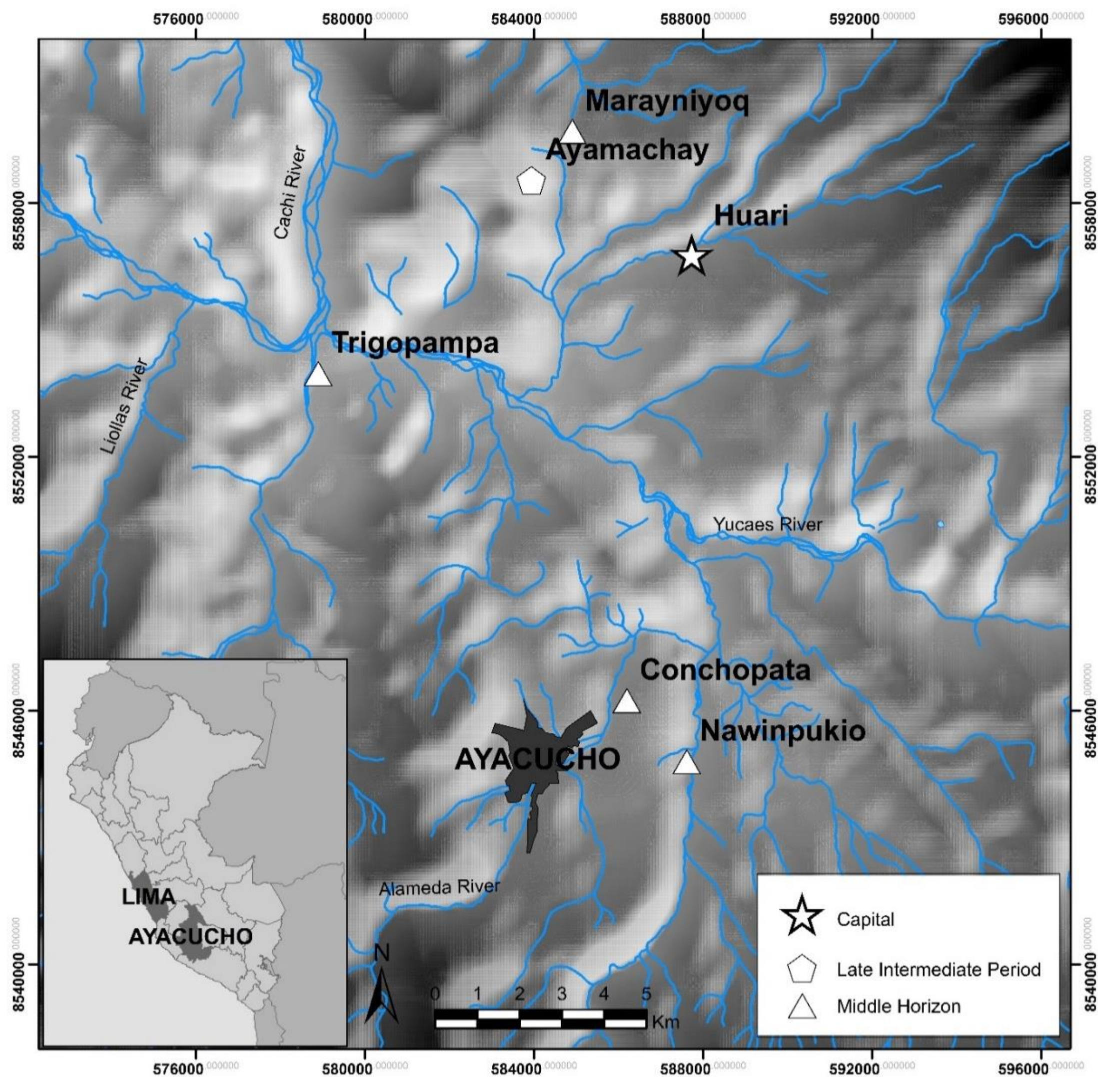


Image courtesy of Tiffiny Tung

For reasons that are not yet fully understood, Wari society began to decline at the end of the first millennium (1000 CE). This decline is documented by the disappearance of Wari-style pottery and textiles, as well as the deterioration of trade networks throughout the Ayacucho Basin (Tung 2012). In contrast to earlier periods of Wari history, poor

nutrition and risk of traumatic injury became remapped across social, political, and geographic spheres, and began to affect a much larger segment of people in some areas. The time following the decline of the Wari, known as the Late Intermediate Period (LIP, 1000 – 1400 CE), was characterized by sociopolitical and ecological volatility in what was previously the Wari heartland, in which various polities of the central Andes struggled to gain control of the region (Arkush 2005, Tung et al. 2008, Arkush and Tung 2013). Changes in architecture, including decreases in monumental construction and increases in defensible site locations and fortifications, signaled a cultural shift in which warfare and violence became more common across certain social and material landscapes (Tung et al. 2016). Coinciding with the period of Wari decline were changes in the environment, including long periods of drought (Bird et al. 2011, Thompson et al. 2013). Some scholars have suggested that environmental changes were the determining factors in the ultimate decline of the Wari state. However, Tung has shown that high levels of violence were characteristic of some terminal Wari sites in the Wari heartland prior to the onset of droughts (Tung 2016, unpublished). This indicates that environmental change alone is not sufficient to explain rates of violence at Huari in the post-Wari (LIP) era, and that there is a more complex set of sociopolitical factors that may influence patterns of violence that have been observed across the Wari to post-Wari transition.

In this study, we examined how cytosine methylation varied between Wari and post-Wari times, where dramatic changes in quality of life were shaped in part by sociopolitical and environmental change. We focus on three archaeological sites spanning Wari era. The first is Conchopata, a Wari-era community in the Wari heartland that was occupied during the height of the Wari culture. Two mortuary sectors at the site of Huari, Cheqo Wasi and Vegachayoq Moqo, are mortuary sectors located in the city of Huari, the

large metropolitan epicenter of the Wari state. To reduce confusion between the terms "Wari" and "Huari", we will follow Isbell's (2004) nomenclature, which uses "Wari" to refer to the culture and cultural material, and "Huari" to refer to the archaeological locality.

The Cheqo Wasi mortuary sector includes burials that were interred during the terminal Wari period, just prior to the final decline of the Wari state (Tung 2014). The Vegachayoq Moqo mortuary sector includes disremembered corpses that were discarded into a pit some 300 years after the decline of the Wari state (Tung et al. 2017). Reconstructing cytosine methylation patterns in aDNA from these three sites provides a way to assess whether epigenetic patterns reflect differences in sociopolitical and environmental conditions in the Wari heartland. In the overviews of the archaeological localities that follow, I employ the various nomenclature regarding age categories (subadult, adult, etc.), status indicators (elite, intermediate elite, or non-elite), and community belonging (social insider or outsider) used in the various bioarchaeological studies pertaining to those sites.

Conchopata (Middle Horizon, 600-1000 CE)

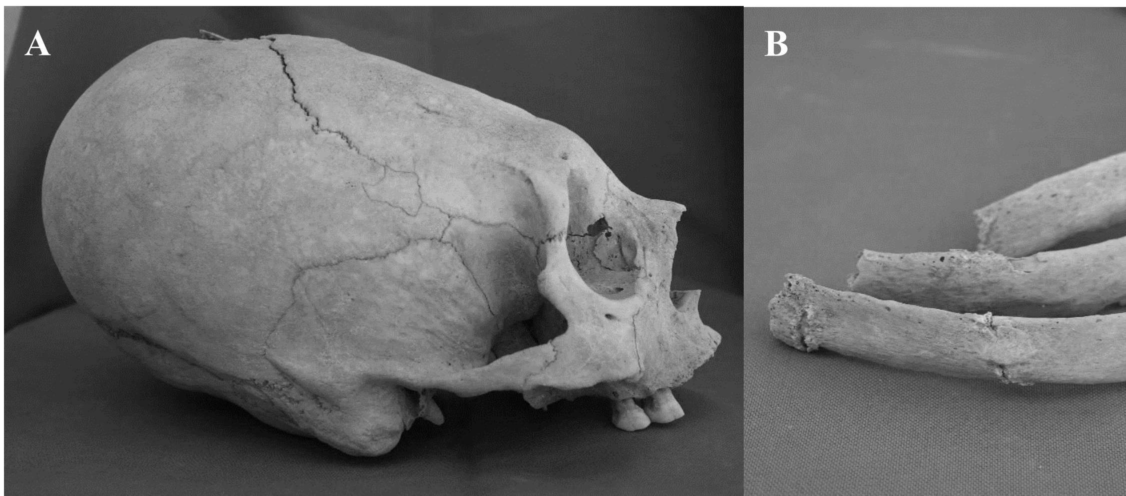
Conchopata is a Wari-era settlement that was occupied during the height of Wari culture. There has been some debate among archaeologists about the sociopolitical organization that may have existed at the site. Some have suggested that aspects of the architecture at Conchopata are best interpreted as royal palaces, and that the site may have been inhabited by a segment of Wari elite (Isbell 2004). However, others have noted that while there are similarities in mortuary styles and practices between Conchopata and the nearby capitol city of Huari, the tombs at Conchopata did not reach the size and grandeur of the royal tombs seen at Huari (Tung and Cook 2006). Therefore, some archaeologists have argued that the community at Conchopata was inhabited not by members of the Wari

elite, but by intermediate elites (Tung and Cook 2006). This latter interpretation is supported by a recent trace element analysis of obsidian artifacts excavated from Conchopata (Burger et al. 2016). This analysis has shown that all the volcanic glass at Conchopata was quarried from local sources in the surrounding Ayacucho basin, indicating that Conchopata was not an urban cosmopolitan center in the same way that the nearby capitol of Huari was.

Extensive bioarchaeological analyses have been conducted at Conchopata. Osteological analyses of antemortem trauma indicate that traumatic violence was structured by social differences in Wari society. No osteological evidence of violence is present among children, suggesting that the skeletal injuries seen among intermediate elites were accrued during adulthood. Twenty-nine percent of intermediate elite men show combat-related skeletal injuries, while 20% of intermediate elite women exhibit injuries on the posterior cranium. While the rates of violence were not statistically significant between women and men, bioarchaeologists have shown that the contexts of violence were different, where men were often facing their attacker(s) while women never were (Tung 2014). In contrast to the intermediate elites, some non-elite females interred at Conchopata show distinct patterns of extreme and recurrent injuries (Tung et al. 2008). For example, an old woman (individual EA1) was exposed to extensive violence throughout her life, with both fully healed and partially healed cranial wounds that showed signs of secondary infections, multiple postcranial fractures including six broken ribs, and antemortem loss of anterior dentition, which were potentially lost due to facial trauma (Figure 3.4, Tung 2014). In addition, this woman exhibited other injuries that are suggestive of hard physical labor, including metacarpal fractures and multiple compression fractures of the lumbar vertebrae (Tung 2014). The extensive damage to her body indicates that she was treated very

differently from other members of the community at Conchopata. However, none of the trauma observed among intermediate elites or non-elites were perimortem injuries, indicating that violence observed among people buried at Conchopata was non-lethal (Tung 2012).

Figure 3.4: Cranial and post-Cranial Trauma observed in individual EA1 from Conchopata.



A: Cranium of an old woman (EA1) with the annular form of cranial modification, where the head is elongated from front to back. Cranial injuries include fully healed and partially healed wounds with signs of secondary infection, and possible antemortem loss of anterior dentition. **B:** Examples of partially healed rib fractures, one of many postcranial wounds observed on the remains of individual EA1. Images courtesy of Tiffiny Tung.

Social outsiders at Conchopata, including non-locals and low-status people, are marked by distinct osteological and mortuary features compared to intermediate elites at the site. Intermediate elites at Conchopata were buried together under house floors with numerous grave offerings (Figure 3.5, Tung 2012). Analyses of strontium isotopes among intermediate elites indicate that they consumed local sources of strontium throughout their

lives, and were likely born in the Wari heartland (Tung and Knudson 2008). In contrast, many sacrificed individuals are interred near D-shaped ritual structures, separate from other burials. While their postcrania were disarticulated, burned, and chopped, the heads of sacrificed people were sometimes transformed into trophies. Sixty percent of the trophy heads show non-local strontium isotope signatures, indicating that some of these individuals had lived outside the Ayacucho Basin during part of their lives (Tung and Knudson 2008). Another adolescent female who was sacrificed was buried alone with sparse grave goods near the D-shaped ritual structure where multiple trophy heads had been deposited (Figure 3.6). Previous analyses of her strontium isotopes indicate that she was non-local, but arrived after the age of 5, and may have been captured during Wari raids of other Andean communities (Tung 2014). Thus, while intermediate elites appear to be locals, sacrificed people with distinct mortuary treatment are largely outsiders. In addition, the old woman with extensive evidence of trauma received distinct mortuary treatment and was buried alone with only a ceramic fragment under her head. While her strontium isotope ratios indicate that she was born in the Wari heartland, she is the only woman in the community to exhibit the annular form of cranial modification, in which the skull is wrapped during childhood to elongate the cranium from front to back (Figure 3.4). Most other women either exhibit no cranial modification, and those that do have modified crania show the fronto-occipital style (Tung 2014). Based on her osteological and mortuary features, it is likely that this low-status woman was viewed as a social outsider at Conchopata. Thus, bioarchaeological evidence indicates that lived experiences of violence at Conchopata were shaped by status, gender, and ethnic differences as well as group belonging in Wari society. However, while violence was shaped by social differences, dietary patterns at Conchopata do not appear to have been significantly stratified along

status, gender, or ethnic lines. Rather, studies of stable carbon isotopes have shown that most people living at Conchopata had access to carbon-enriched foods such as maize (Finucane et al. 2006), and social outsiders do not appear to have substantially differed from intermediate elites at the site.

Figure 3.5: Individual EA205 *in situ*, illustrating the mortuary context of an intermediate elite individual at Conchopata.

Intermediate elites at Conchopata were interred under house floors with grave offerings near other intermediate elites. Image courtesy of William Isbell and Anita Cook.

Figure 3.6: Mortuary context of EA20, an adolescent female who was sacrificed and interred near D-shaped ritual structure at Conchopata.

This adolescent female was ritually sacrificed and then buried alone without grave offerings. Image courtesy of William Isbell and Anita Cook.

Huari, Cheqo Wasi Sector (Terminal Wari, 1000-1100 CE)

Ten kilometers to the north of Conchopata is the city of Huari, the large cosmopolitan capitol of the Wari state throughout the Middle Horizon. Huari was occupied before, during, and after the decline of the Wari culture, and burials from various time periods were interred in different mortuary sectors of the city. The mortuary sector of Cheqo Wasi contained burials from the latest phase of the Wari state, known as terminal Wari (Figure 3.7, Tung 2014). Cheqo Wasi was one of the most elaborate funerary sectors at Huari (Tung 2014), and is characterized by the presence of monumental architecture

including an elaborate stone arch and large royal tombs (Figure 3.7). These architectural and mortuary features suggest that the highest strata of society were being interred there before the eventual decline of the Wari (Tung 2014). While this site was repeatedly looted during pre-Hispanic times and again following European colonialism, architectural and osteological analyses of Cheqo Wasi provide the only known picture of the life and health of capitol elites during the final phases of the Wari state (Tung 2014).

Figure 3.7: Mortuary complex in the Cheqo Wasi sector of Huari.

A: Tombs of Cheqo Wasi. **B:** An example of a megalithic tomb at Cheqo Wasi. Images courtesy of Tiffiny Tung.

Among the various mortuary sectors that have been studied at Huari at so far, osteological analysis has shown that people interred at Cheqo Wasi suffered the greatest rates of traumatic violence. In all, 50% of individuals show some evidence of cranial trauma and the vast majority of these injuries were non-lethal (42% antemortem and 8% perimortem, Tung 2014). While it is unusual to observe violence among children in the Andes, 66% of subadults at Cheqo Wasi showed evidence of cranial trauma (Tung 2014). Sixty-four percent of women show signs of cranial trauma, with a more posterior than anterior distribution of injuries suggesting that their injuries were often accrued while the women were not facing their attacker (Tung 2014). Fifty percent of men show signs of cranial trauma, and in contrast, their injuries show a more anterior than posterior distribution, indicating that they were more often facing their attacker than the women were (Tung 2014). No significant sex-biased differences have been observed in overall rates of trauma at Cheqo-Wasi. Importantly though, a higher fraction of women overall show cranial trauma relative to men, while men tend to show more cranial traumas per individual. In addition to the head injuries at Cheqo-Wasi, 18% of left ulnae have parry fractures that can be sustained when someone blocks an oncoming attack with their arm. This rate of parry fractures is the highest that has been observed at any Wari-era site (Tung 2014).

Compared to sites that date from the height of the Wari state, such as Conchopata, traumatic violence was much more common among those interred in the Cheqo Wasi mortuary sector of Huari as the Wari era was coming to a close. While no violence was observed among any of the children at Conchopata, the majority of children that have been studied at Cheqo Wasi suffered traumatic injuries. Among adults at Cheqo Wasi, cranial injuries were larger and far more numerous than those observed at Conchopata, and more individuals show signs of repetitive injuries (Tung 2014). Taken together, osteological

evidence indicates that people interred at Cheqo Wasi near the decline of the Wari state were more likely to be the victims of traumatic violence than those who lived during the height of Wari society (Tung 2014). This could indicate that patterns of violence were changing throughout the Wari-era, or that engaging in violent acts was a means of establishing and/or maintaining elite status in Wari society, as those who engaged in violence and survived were interred in the most elaborate mortuary contexts (Tung 2014). These interpretations are not mutually exclusive, and one or both of these factors could be explanations for the dramatic increase in violence observed at Cheqo Wasi relative to other Wari-era sites. In spite of the high rates of violence, stable isotope analyses indicate that elites living at Cheqo Wasi had higher access to carbon-enriched foods during the terminal Wari era than others who were interred at Huari after the decline of the Wari civilization.

Huari, Vegachayoq Moqo Sector (Post-Wari, 1350 CE)

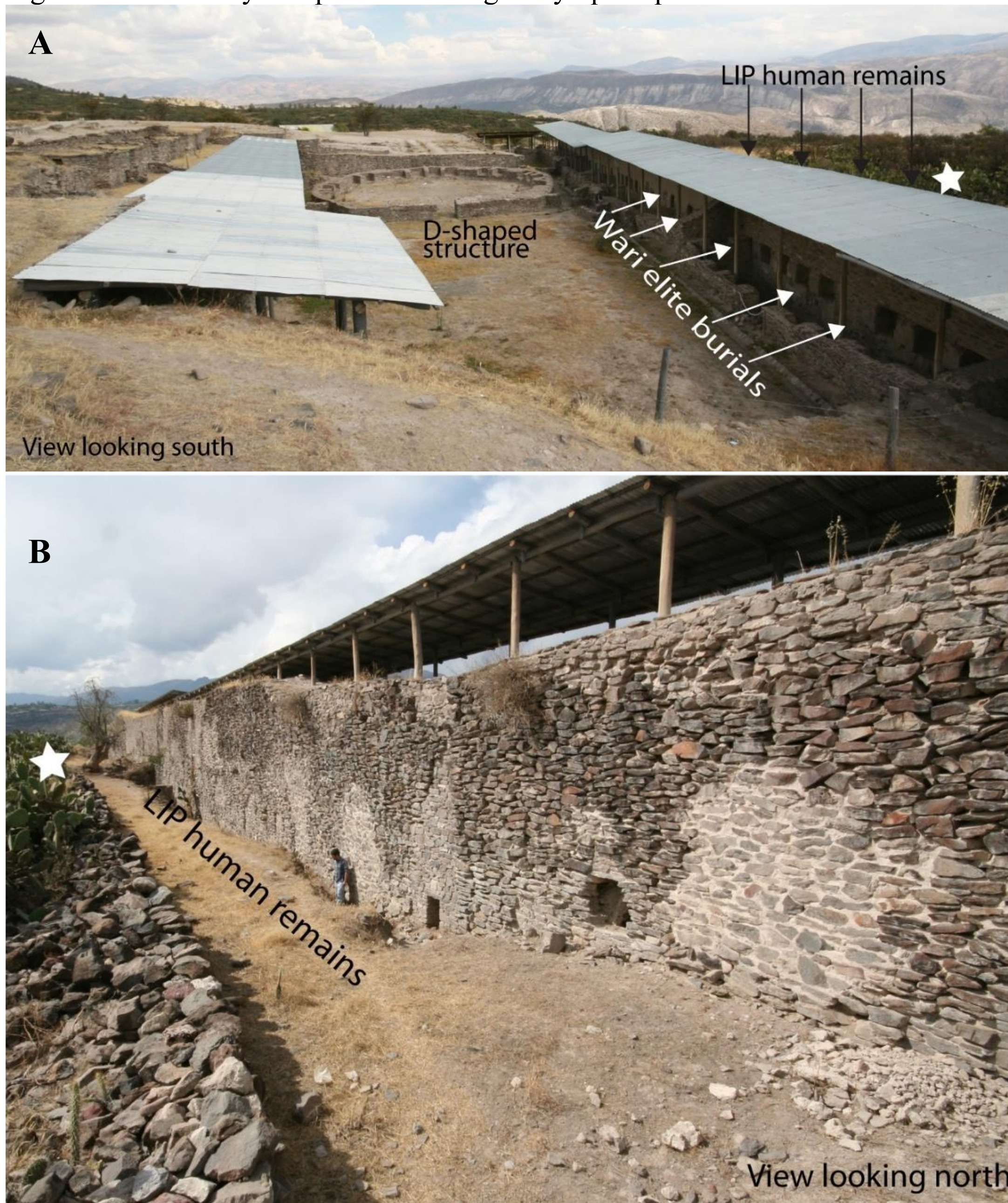
The time following the decline of the Wari state, known as the Late Intermediate Period (LIP), was characterized by large scale changes in settlement patterns and material culture throughout the Ayacucho Basin. These changes included the disintegration of state control in the former Wari heartland, the decline and or reorganization of ceramic and textile production infrastructures and trade networks, and shifts in occupation patterns from the valley floors to higher-elevation sites (Covey 2008, Tung 2016). Changes in political organization are observed in the depopulation of sites in the Wari heartland as well as the decline in construction of monumental architecture (Covey 2008, Finucane 2009, Arkush and Tung 2013, Tung 2016). Increases in social unrest and conflict in some areas is reflected through the increase in fortifications (Covey 2008, Arkush and Tung 2013). Shifts in diet and patterns of violence also changed dramatically in the LIP compared with the height of the Wari state in the MH. In particular, rates of perimortem violence dramatically

increased compared to both Conchopata and Cheqo Wasi. There are likely many interacting factors that influenced the increases in violence observed at various archaeological sites across the Ayacucho Basin during the LIP, including the decline of the Wari state, long periods of drought that may have led to increased resource competition and conflict, and/or changes in social and political structures that may have altered the contexts and social meaning of violence (Tung 2016).

At Huari, the mortuary sectors of Vegachayoq Moqo and Monqachayoq provide important insights into life at the former capitol following the decline of Wari society. Huari was either reoccupied or used for mortuary purposes during the LIP, and bioarchaeological analyses of human remains have helped to shed light on the aftermath of the Wari decline and its effects on people's diets and relative burdens of violence in the former Wari heartland (Tung 2016). At Vegachayoq Moqo, the mortuary context is quite different than those observed at the earlier, Wari-era sites of Cheqo Wasi and Conchopata (Figure 3.8). Archaeologists working at Vegachayoq Moqo have excavated mass graves containing thousands of comingled remains of more than 100 people (Bragayrac 1991, Tung 2016). Eighty percent of the long bones show evidence of cut marks, indicating that the bodies had been dismembered before being thrown into a trench that ran along a wall beneath a D-shaped ritual structure (Tung 2016, Figure 3.8). In addition, while signs of antemortem and perimortem trauma among children are rarely observed in the Andes, children interred at Vegachayoq Moqo appear to have died violent deaths, underscoring the social and political volatility of this area during LIP (Tung 2016). While no antemortem trauma was observed on the remains of these children, rates of perimortem trauma were very high. Sixty-six percent of the children interred at Vegachayoq Moqo show signs of having suffered perimortem cranial fractures, which suggests changes in the treatment of

children in the former capitol city during the tumultuous era following the decline of Wari society (Tung 2016). Osteological analysis has shown that these perimortem blows to the head were intentional and systematic, largely resulting from blunt force traumas to the left posterior sides of the crania (Tung 2016). Rates of violence were also high among children interred at the nearby mortuary sector of Monqachayoq, affecting 30% of the children there (Tung 2008). In addition, nutrition among children appears to have changed in this region during post-Wari times. Analysis of stable Carbon isotopes has shown that children in the post-Wari era had significantly lower $\delta 13C$ values, indicating that they had less access to carbon-enriched foods like maize relative to children in the Wari period (Tung 2016).

Figure 3.8: Mortuary complex in the Vegachayoq Moqo sector of Huari.



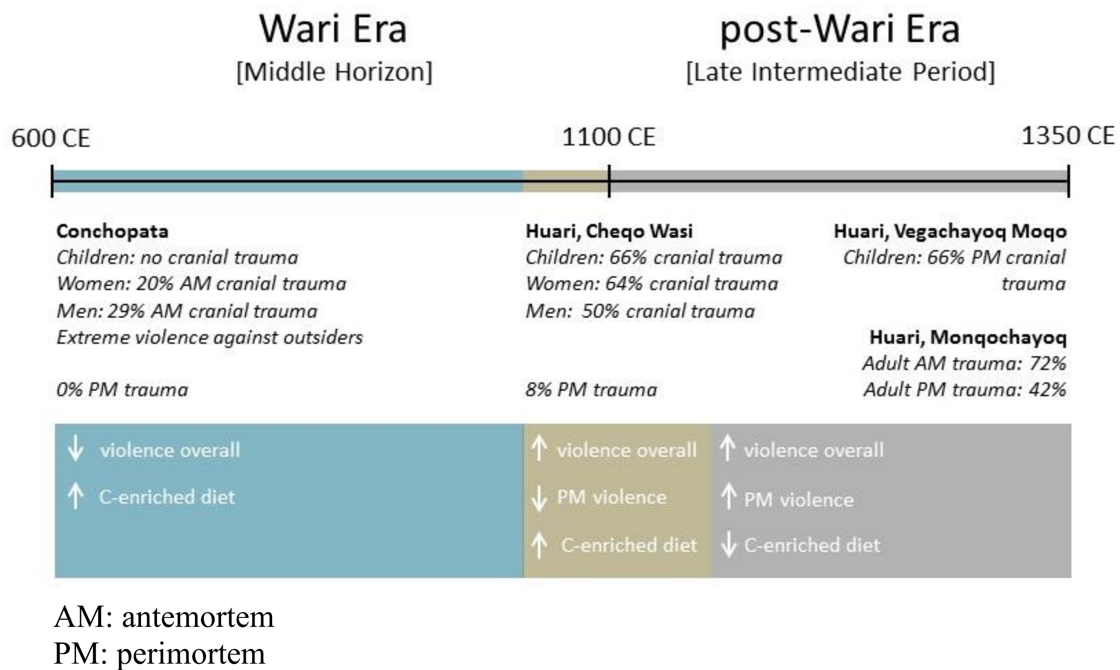
A: Vegachayoq Moqo complex with D-shaped ritual structure that was used during the Wari era. **B:** Wall running along the western margin of the Vegachayoq Moqo complex just below the D-shaped structure, where bodies from the LIP were deposited in a commingled grave. Images courtesy of Tiffiny Tung.

While osteological analyses of adults interred at Vegachayoq Moqo is still ongoing, the remains of adults interred at the nearby and contemporaneous mortuary sector of Monqachayoq may serve as a useful proxy for rates of violence among adults interred at Huari during the LIP. Here, antemortem trauma affected 71% of adults. In addition, perimortem cranial fractures were observed on 42% of adults, indicating a dramatic increase in lethal attacks relative to Wari-era sites (Tung 2008, Kemp et al. 2009). The rates of antemortem violence are far higher than those observed at Conchopata during the height of Wari society, and rates of both antemortem and perimortem trauma far surpass those observed in the terminal Wari period at the mortuary sector of Cheqo Wasi.

For the three archaeological sites represented in this study, it is clear that violence affected many more individuals at the terminal and post-Wari sites than at the Wari-era sites, and that access to carbon-enriched foods declined at the post-Wari site (Figure 3.9). These changes were shaped at least in part by sociopolitical and environmental changes in the Ayacucho basin across the Wari to post-Wari transition. The rich history and bioarchaeology of Conchopata, Cheqo Wasi, and Vegachayoq Moqo provide an ideal context for assessing whether these changes are associated with epigenetic differences in aDNA. In living humans, LINE-1 methylation has been shown to be affected by factors such as dietary, psychosocial, and structural inequalities (Zhang et al. 2011a, Zhang et al. 2011b), all of which are characteristic of the Wari archaeological record to varying degrees. Therefore, we obtained skeletal and dental samples from 33 individuals living before and after the Wari decline and evaluated patterns of LINE-1 methylation. Based on the mortuary and osteological evidence from Conchopata, we hypothesized that low status women, including those who experienced repetitive trauma and outsiders who were ritually sacrificed, would show different patterns of LINE-1 methylation compared to intermediate

elites at the site. Taking the bioarchaeological evidence for Conchopata, Cheqo Wasi, and Vegachayoq Moqo together, we hypothesized that global genomic methylation of LINE-1 would decrease from Wari to post-Wari times, as access to carbon-enriched foods diminished and exposure to violence worsened for the people represented in this study. We also hypothesized that variability in percent methylation would increase as Wari social orders disintegrated, adversity became more pronounced, and vulnerability became more erratically structured in the population. Here, we provide the first study of whether ancient methylation patterns track environmental and sociopolitical changes in the ancient world.

Figure 3.9: Overview of violence and diet at Wari and post-Wari sites.



AM: antemortem
PM: perimortem

MATERIALS AND METHODS

Tissue Samples

To assess the LINE-1 methylation patterns of Wari and post-Wari populations, we obtained 33 dental and skeletal samples from three archaeological sites in the Ayacucho Basin, Peru. From the Wari era, we collected skeletal samples from the site of Conchopata (N=14). From the terminal Wari era, we collected skeletal samples from the Cheqo Wasi mortuary sector of Huari (N=9). Finally, from the post-Wari era we collected samples from the Vegachayoq Moqo mortuary sector of Huari (N=10). Osseous samples were exported with support for genetic research from the provincial office in Ayacucho and the Ministry of Culture in Lima, Peru.

To evaluate whether LINE-1 methylation values observed in teeth are correlated with those in other tissues, we analyzed four different tissues from two living individuals. One individual had experienced physical violence throughout life, while the other had not. It is not currently known whether other significant aspects of life history differ between the two individuals. Third molars, whole blood, buccal cells, and hair were collected from both individuals with informed consent (UT IRB 2012-05-0105). Molars were collected opportunistically following routine dental extractions unrelated to the research. Approximately 50 microliters (µL) of whole blood was collected via finger pricks using retractable lancets and placed in a 1.5 mL tube containing 150 µLs of RNAlater® (ThermoFisher). Buccal cells were collected using sterile swabs and hair samples were collected by cutting 2-3 cms off of the distal end without attached follicles.

DNA Extraction

To extract DNA from contemporary tissues, three tissue-specific protocols were used. Negative control extractions (blanks) were included to monitor for possible

contamination. DNA was obtained from contemporary teeth following the method described in Dabney et al. (2013). Prior to DNA extraction, teeth were decontaminated by immersing in 6% sodium hypochlorite (full strength bleach) for 10 minutes and then rinsing twice with DNA-free water. Teeth were then UV irradiated in a 254-nm emitting DNA crosslinker for 5 minutes on each side. Rather than drilling bone powder from the tooth root, we modified the Dabney et al. (2013) approach for a minimally destructive extraction protocol (following Bolnick et al. 2012) where the whole tooth was immersed in a digestion buffer containing 4,937.5 µL of 0.45 M EDTA and 62.5 µL of 20 mg/mL proteinase K and placed on a thermal rocker at 37 ˚C for 18 hours. In addition, we decontaminated the guanidine hydrochloride binding buffer by adding 100 µL of a silica dioxide suspension described in Rohland and Hofreiter (2007) and placing on a rocking platform at room temperature for 18 hours. Buccal swabs and 100 µL of each whole blood sample were extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Hair samples were decontaminated by immersing in a 3% sodium hypochlorite solution for 10 minutes, and then rinsing three times with DNA-free water. Hair samples were then extracted using the DNeasy Blood and Tissue kit (Qiagen), following the user developed protocol for the purification of total DNA from nails, hair, or feathers (Protocol: DY04 Aug-06).

To extract DNA from archaeological samples, teeth and bone samples were first immersed in 6% sodium hypochlorite (full strength bleach) for 10 minutes and then rinsed twice with DNA-free water. Samples were then UV irradiated in a 254-nm emitting DNA crosslinker for 5 minutes on each side. For skeletal material, we gently abraded the surface of the sample with a dental drill fitted with a 0.5 mm diamond burr and then drilled 150-200 mg of bone powder from which we extracted DNA following the method described in

Dabney et al (2013), with the addition of purifying the binding buffers with a silica dioxide suspension as described above. For dental samples, we modified the Dabney et al. (2013) method for minimally destructive extraction as described above.

Mitochondrial DNA Analysis

To assess the preservation of endogenous DNA in each of the archaeological samples, we analyzed a portion of the first hypervariable region (HVR1) of the mitochondrial DNA (mtDNA) and looked for diagnostic mutations commonly found among Native Americans prior to the arrival of Europeans. We PCR amplified short overlapping fragments of HVR1 following the method described in Bolnick et al. (2012). To confirm amplification, PCR products were visualized via gel electrophoresis using 5X GelRed™ solution (Biotium) and a 6% polyacrylamide gel. Confirmed PCR amplicons were diluted 1:10 in DNA-free water and submitted for PCR purification and Sanger sequencing at the Core Sequencing Facility at the University of Texas at Austin. Resulting sequences were edited using Sequencher v. 5.3, and were aligned to the Cambridge Reference Sequence to identify sequence differences using a custom function in the [R] statistical environment (R Core Team 2017). Haplotypes and haplogroups were determined via MitoTools, using the PhyloTree mtDNA database (build 17).

Bisulfite Conversion and Pyrosequencing

DNA extracts from contemporary molars, whole blood, buccal cells, and hair were quantified with a NanoDrop spectrophotometer and input volumes for bisulfite conversion protocols were adjusted so that DNA concentrations were standardized across all reactions. For archaeological samples, 20 μL of each extract was included in bisulfite conversion reactions. Ancient and contemporary tissues were bisulfite converted using the EpiTect

Fast Bisulfite Kit (Qiagen) following the manufacturer's protocol for low concentration samples. We modified the bisulfite conversion protocol following Smith et al. (2015), Specifically, we UV irradiated buffers BL (31 mL), BW (13 mL concentrate), BD (3 mL concentrate), and EB (15 mL) for 15 minutes in a 254-nm emitting DNA crosslinker to eliminate any background contaminants that might be present in these reagents. We also omitted the carrier RNA, as previous use of this reagent in aDNA applications has shown trace levels of DNA contaminants.

Following bisulfite conversion, we PCR amplified a 108 base pair (bp) region of the human long interspersed nuclear element (LINE-1) promoter that contains a total of eight CpG sites, following the method described in Ricceri et al. (2014). PCRs were prepared with 1 µL of bisulfite product in a 25 µL total reaction volume using the Amplitaq Gold™ kit (ThermoFisher). Each reaction also included 0.78 µL of 20 mg/mL BSA (Roche) and 1.5 µL of MasterAmp™ 10X PCR enhancer with betaine (Epicentre). To confirm amplification, PCR products were visualized via gel electrophoresis using 3X GelRed™ solution (Biotium) and a 6% polyacrylamide gel. Confirmed PCR amplicons were submitted for pyrosequencing on Qiagen's Q96 platform at the BASiC Core Facility at the University of Texas San Antonio Health Science Center. Percent methylation, or the fraction of cytosines that were methylated at each CpG position across LINE-1 amplicons, was calculated for four CpG positions.

Authentication

Ancient DNA obtained from archaeological remains is highly degraded and requires strict precautions to minimize contamination from exogenous sources of DNA and authenticate results (Kaestle and Horsburgh 2002, Paabo et al. 2004, Gilbert et al. 2005, Willerslev and Cooper 2005, Shapiro 2012). All phases of work with archaeological

samples, including aDNA extractions, bisulfite conversions, and PCR setups were conducted in the aDNA facility at the University of Texas at Austin. The aDNA facility is a restricted-access, positive air pressure, HEPA-filter ventilated space with overhead UV-irradiating lights that is dedicated to pre-PCR analyses of aDNA. The post-PCR facility is located in a separate building, and all movement of materials and personnel was unidirectional (from pre-PCR to post-PCR facility) to prevent contamination from highly concentrated, post-PCR amplified DNA. Additional precautions included the use of sterile and disposable hooded coveralls, hair covers, face masks, sleeve covers, dedicated shoes, and two pairs of gloves. We frequently decontaminated laboratory benchtops and equipment with 6% sodium hypochlorite (full strength bleach), and decontaminated the entire lab space weekly with a 3% sodium hypochlorite solution (1:1::bleach:water, v/v). We also irradiated the facility with a 254-nm emitting overhead UV light for 12 hours following each use, while tubes, containers, and reagents were UV irradiated (when possible) in a 254-nm emitting DNA cross-linker for 15 minutes prior to use.

Negative controls included DNA extraction blanks, conversion blanks (bisulfite conversion reaction mixtures containing no DNA), and PCR negatives to identify the presence of any contamination at each stage of sample analysis. We performed 2-3 independent PCR amplifications for each aDNA extract, and additional PCR amplifications were performed using an independent DNA extraction for a subset of individuals (N=3) to verify the authenticity of the results. Many of the archaeological samples used in this study were previously extracted in an independent lab, and our mtDNA results were verified against previously published sequences (Kemp et al. 2009).

During pyrosequencing, the dispensation program included bisulfite control positions to monitor the efficiency of bisulfite conversion in each individual sample.

However, because pyrosequencing primers are designed to amplify bisulfite converted sequences, bisulfite products may be preferentially amplified over unconverted DNA, limiting the accuracy of bisulfite control dispensation as a measure of conversion efficiency. To better evaluate the efficiency of the bisulfite conversion process, three aDNA samples were bisulfite converted a second time to assess the reproducibility of the methylation assays across independent conversions.

Statistical Analyses

In this study, we conducted two sets of statistical analyses. First, we assessed methylation of the LINE-1 promoter region in four tissues from living humans. Because CpG positions 3-6 showed evidence of polymorphism that possibly contributed to variability in the estimation of percent methylation at those positions, only four CpG positions (CpGs 1, 2, 7, and 8) were analyzed. To evaluate whether LINE-1 methylation patterns in contemporary teeth are consistent with methylation patterns observed in buccal cells, blood, and hair, we performed both one-way and two-way ANOVAs. One-way ANOVAs were first used to test whether percent methylation values observed in teeth were significantly different from percent methylation observed in the three other tissues. This test allowed us to assess differences in the numerical response parameter of percent methylation relative to the categorical parameter of different tissue types. We then performed two-way ANOVAs to assess the relative effects of CpG position and tissue type across all 4 CpG positions included in this analysis. These tests allowed us to assess whether methylation levels were shaped by the CpG position, by the tissue of origin, or by an interaction between CpG position and tissue source. If methylation is different across the four tissue types tested here, we would expect two-way ANOVAs to identify tissue source as the main effect shaping methylation patterns. If methylation levels are the same

no matter what the tissue source is, we would expect CpG position to be the main effect shaping methylation patterns. If both tissue source and CpG position influence methylation patterns, we expect that there will be a significant interaction between the two main effects included in the two-way ANOVA tests. Finally, we used paired t-tests to detect any significant differences in methylation at each CpG position between individuals one and two. Shapiro-Wilk tests, quantile-quantile plots, and histograms showed that percent methylation data for each CpG position did not violate the assumption of normality required for parametric statistical analyses.

Secondly, we evaluated methylation of the same 4 CpG positions of the LINE-1 promoter region in aDNA obtained from human remains interred at Conchopata, Cheqo Wasi, and Vegachayoq Moqo. To test the hypothesis that social outsiders at Conchopata had different methylation patterns than local intermediate elites, we conducted pairwise Wilcoxon rank-sum tests between all individuals in the population, using Holm-Bonferroni correction for multiple comparisons (Holm 1979). Shapiro-Wilk tests, quantile-quantile plots, and histograms showed that percent methylation data for each CpG position violated the assumption of normality required for parametric statistical analyses. Therefore, non-parametric statistical comparisons were used in this case. CpG positions 1, 7, and 8 showed some stochasticity between independent pyrosequencing replicates for one or more individuals. Therefore, Wilcoxon rank-sum tests were only conducted using percent methylation data from the second CpG position, which showed the least stochasticity between independent pyrosequencing replicates across all individuals from Conchopata. This approach allowed us to conduct the most conservative statistical test possible while maximizing the sample size. Next, to assess whether there were statistical differences between archaeological localities in percent methylation values at each of the CpG sites,

we conducted four one-way ANOVAs comparing methylation levels at each CpG site between Conchopata, Cheqo Wasi, and Vegachayoq Moqo. To assess whether there was greater variance between individuals in post-Wari times relative to Wari times, we grouped the percent methylation data into Wari and post-Wari cohorts and calculated the variance at each CpG position for all individuals in each group. We then conducted a one-way ANOVA to test whether variance was significantly different between Wari and post-Wari times. We also grouped the variances in percent methylation by archaeological locality and conducted one-way ANOVAs to test for any differences between sites. Because previous work has shown that variance between pyrosequencing replicates is inversely related to DNA preservation (Smith et al. 2015), we controlled for the influence of DNA degradation in our results by conducting a second round of each statistical test outlined above. The second set of statistical analyses included only the subset of methylation data that showed lowest variability between independent measures of percent methylation for each individual. The results of these secondary tests did not show any significant differences from the initial rounds of statistical testing that were based on all available data, and did not affect our interpretations. All phases of statistical analysis for modern and aDNA were conducted in [R] using the packages lsr, dplyr, and tidyr, and graphical outputs were generated using the package ggplot2.

RESULTS

LINE-1 Methylation in Contemporary Human Tissues

Because methylation patterns can vary between tissues, we conducted a preliminary analysis of LINE-1 methylation patterns in four tissues from two living people to evaluate how methylation patterns in archeologically available tissues (such as teeth and hair) compare with more peripheral tissues that have been commonly studied in living populations (such as buccal cells and blood). We successfully characterized LINE-1 methylation in all tissues tested from living humans (Table 3.1). Our one-way ANOVA analyses showed that percent methylation values observed in molars were statistically indistinguishable from buccal cells and whole blood from both of the living individuals tested (Table 3.2). For individual two, LINE-1 methylation values observed in teeth and hair were also not statistically different. However, for individual one, the difference in LINE-1 methylation between molars and hair reached statistical significance (p=0.049).

Table 3.1: Percent methylation of LINE-1 from multiple tissues in living humans.

Individual	Tissue	Percent Methylation			
		Pos. 1	Pos. 2	Pos. 7	Pos. 8
1	Molar 1	61.3	36.9	57.1	29.1
	Molar 2	63.8	37.9	57.9	29.1
	Hair	76.1	44.4	67.9	33.6
	Buccal 1	56.8	34.0	51.5	26.1
	Buccal 2	52.4	32.4	50.9	26.3
	Blood	57.2	34.5	55.5	28.1
2	Molar	68.3	39.4	60.1	31.1
	Hair 1	73.4	49.0	58.0	29.7
	Hair 2	80.8	56.8	49.8	27.8
	Buccal	65.8	38.2	58.2	29.9
	Blood	66.6	39.2	60.9	30.8

Table 3.2: Results of one-way ANOVAs between percent methylation values of LINE-1 in four tissues from living humans.

Tissue Comparison	p-values	
	Individual 1	Individual 2
Molar vs. Blood	0.23	0.86
Molar vs. Buccal	0.88	0.79
Molar vs. Hair	**0.049***	0.40

*statistically significant

Two-way ANOVA tests of LINE-1 methylation evaluating the relative effects of CpG position and tissue source showed variable results between individuals one and two (Figure 3.10, Table 3.3). For individual one, the interaction effect between CpG position and tissue source was statistically significant (p=0.017), indicating LINE-1 methylation patterns differ across both CpG positions and tissue sources. However, the main effect of CpG position was far more significant (p=3.33 x 10^{-10}) and CpG position had a much larger effect size than either tissue source or the interaction between CpG position and tissue source (η^2=0.87, 0.11, and 0.017, respectively). Because one-way ANOVA results showed that percent methylation in hair was significantly different from that of molars for individual one, we ran a second two-way ANOVA analysis in which methylation data for hair was removed from the model. In this updated model, the main effect of CpG position remains significant (p=1.063 x 10^{-09}), but the interaction between CpG position and tissue source is no longer significant (p= 0.26). These results indicate that methylation values are similar between molars, buccal cells, and blood, where CpG position predicts methylation levels regardless of tissue source. However, methylation patterns in hair are more distinct and appear to be more tissue-specific. For individual two, the interaction between CpG position and tissue source was not statistically significant (p=0.40), but the main effect of CpG position was highly statistically significant (p=0.00047). As with individual one, CpG

position had a much larger effect size than either tissue source or the interaction between CpG position and tissue source (η^2=0.90, 0.02, and 0.06, respectively). Paired t-tests indicated that individual one was significantly hypomethylated at the first and second CpG positions relative to individual two ($p \leq 0.028$). Excluding methylation data from hair samples, individual one was significantly hypomethylated at all four CpG positions relative to individual two ($p \leq 0.030$).

Figure 3.10: Comparison of LINE-1 methylation levels across four tissues from living humans.

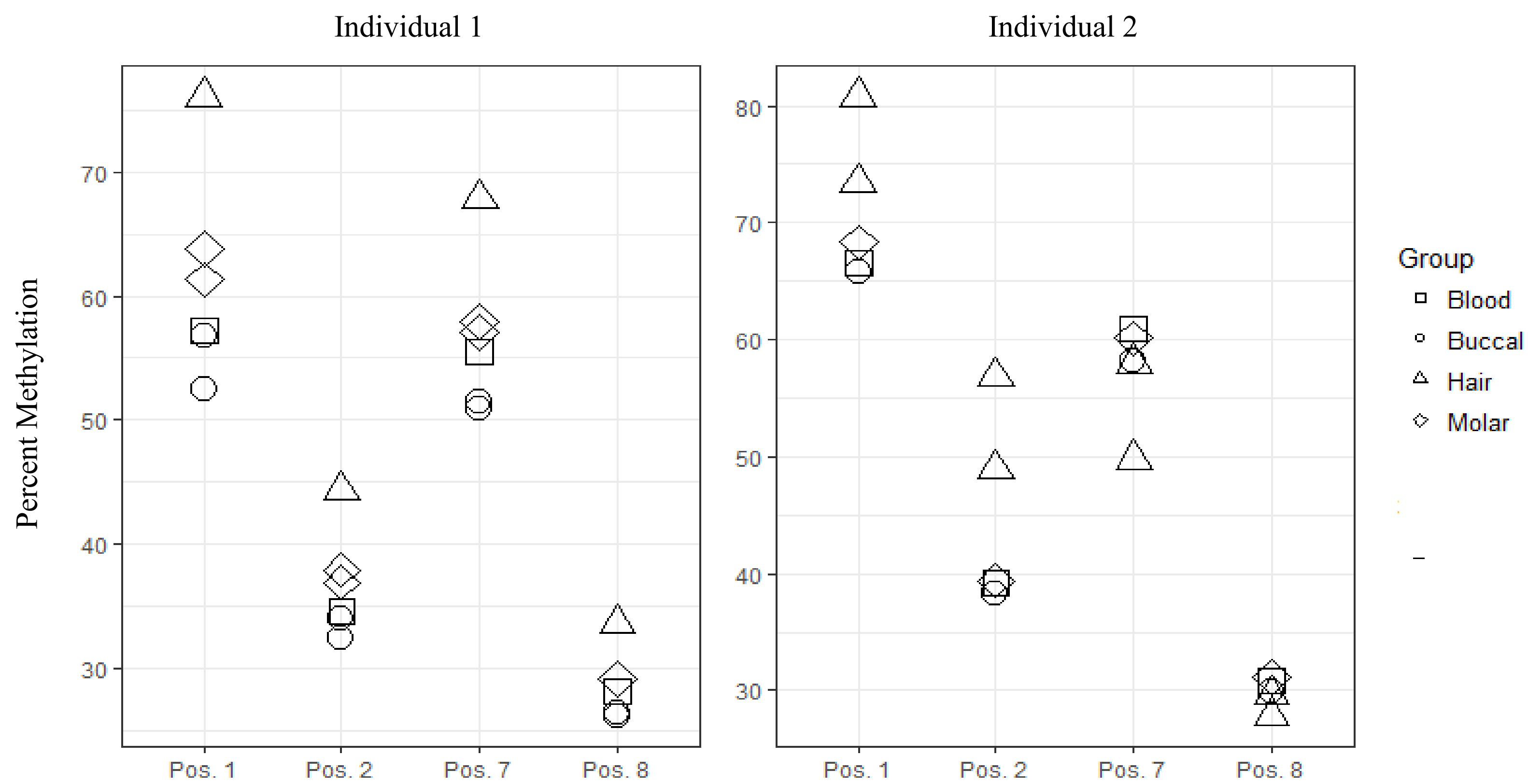


Table 3.3: Results of two-way ANOVAs assessing the effects of CpG position and tissue source in living humans.

Effect	**Individual 1**		**Individual 2**	
	p	**η^2**	**p**	**η^2**
CpG Position	3.3×10^{-10}	0.87	7.2×10^{-4}	0.90
Tissue Source	1.2×10^{-6}	0.11	0.40	0.02
Position:Source	0.017	0.017	0.36	0.06

p: p-value
η^2: effect size

LINE-1 Methylation in Ancient Wari and Post-Wari Populations

Of the 33 Wari and post-Wari skeletal samples that we attempted to obtain aDNA from, a total of 13 had preserved endogenous DNA (success rate of 39%). Of these 13 samples, five are from Conchopata, four are from Cheqo Wasi, and four are from Vegachayoq Moqo. Rates of trauma and stable isotope ratios were determined in previous studies (Table 3.4). Resulting mtDNA sequences correspond to haplogroups that are known to be present in the Americas prior to European colonization (Table 3.5). In addition, for the subset of samples in this study that had been included in other paleogenomic research, haplogroup assignments reported here are consistent with previously published data (Kemp et al. 2009).

Table 3.4: Bioarchaeological data for individuals included in this study.

Sample	Site	Era	Time Period	Sex	Age	δ18O den carbonate	δ13C dent carbonate	δ13C collagen	δ15N collagen	87Sr/86Sr	Skeletal Trauma
EA1	C	W	600-1000 CE	F	Old Adult	-8.65 to -7.5	-2.7 to -5.89			0.70583	AM
EA6	C	W	600-1000 CE	?	Infant	-9.27	-2.1			0.70673	No
EA20	C	W	600-1000 CE	F	Young Adult	-7.0	-6.6			0.71058	No
EA88	C	W	600-1000 CE	?	Infant						No
EA205	C	W	600-1000 CE	?	Infant					0.70572	No
HCW46	CW	TW	1100 CE	M	Adult	-8.8 to -10	-5.4 to -1.1				Unknown
HCW119	CW	TW	1100 CE	F	Young Adult	-0.9 to 1.6	-9.6 to -9.3				AM
HCW358	CW	TW	1100 CE	F	Young Adult	-10.1	-0.1				AM and PM
HCW860	CW	TW	1100 CE	M	Young Adult	-2.6 to -0.74	-9.2 to -8.9				No
HVM11	VM	PW	1350 CE	F	Teen	-8.92	-4.90	-12.07	8.6	0.70622	PM
HVM16	VM	PW	1350 CE	F	Young Adult	-9.38	-5.24			0.70611	PM
HVM19	VM	PW	1350 CE	F	Young Adult			-11.68	16.3		No
HVM66	VM	PW	1350 CE	M	Old adult	-7.9	-3.8	-11.5 to -10.4	9.5 to 13.3		AM and PM

C: Conchopata, CW: Cheqo Wasi, VM: Vegachayoq Moqo, W: Wari, TW: Terminal Wari, PW: Post-Wari, F: Female, M: Male, AM: Antemortem, PM: Perimortem. (Data adapted from Tung 2012 and Tung, in press)

Table 3.5: Mitochondrial DNA results from Wari and post-Wari skeletal remains.

Sample	Haplotype	HG
EA1	16223T, 16325C, 16362C	D1
EA6	16298C, 16325C, 16327T	C1
EA20	16258G, 16357C	B
EA88		C
EA205		C
HCW46	16221T, 16298C, 16327C	C
HCW119	16223T, 16325C, 16362C	D1
HCW358	16217C, 16261T	B
HCW860	16217C, 16319A	B
HVM11	16223T, 16298C, 16325C, 16327T	C1
HVM16	16223T, 16298C, 16325C, 16327T, 16361A	C1
HVM19	16223T, 16287T, 16290T, 16295T, 16296T, 16325C, 16327T	C1
HVM66	16223T, 16325C, 16327T	C1

For the 13 samples that had preserved endogenous mtDNA, we assessed LINE-1 methylation patterns. All 13 samples tested yielded measures of cytosine methylation. However, many of the CpG positions for sample HVM11 failed to produce percent methylation data during pyrosequencing runs and that sample was excluded from subsequent data analysis. The final methylation data included percent methylation from a total of 12 Wari and post-Wari individuals, with five from Conchopata, four from Cheqo Wasi, and three from Vegachayoq Moqo (Table 3.6).

Table 3.6: LINE-1 methylation results from Wari and post-Wari individuals.

Sample ID	CpG 1	CpG 2	CpG 7	CpG 8
EA1	70.2	41.6	62.1	29.7
	61.6	39.2	55.3	29.3
EA6	61.8	34.7	56.5	34.8
	61.4	30.3	56.5	20.4
EA20	76.0	49.7	62.7	9.9
	22.3	48.6	56.6	23.4
EA88	64.9	34.9	56.4	28.6
	64.2	32.4	56.0	25.8
EA205	55.9	35.2	68.9	20.0
	56.0	27.7	49.6	27.8
HVM16	42.0	24.5	30.0	18.0
	43.8	28.0	58.6	26.2
HVM66	40.5	47.0	62.3	26.8
	48.7	37.8	48.7	27.5
HVM19	98.2	73.8	76.9	13.8
	96.3	1.4	83.9	13.2
HCW46	68.4	65.3	45.0	4.4
	27.6	34.2	51.3	41.3
HCW860	70.0	41.5	56.6	29.5
	70.4	41.3	56.9	30.4
HCW119	65.1	37.8	57.7	29.5
	66.7	39.1	58.4	28.6
HCW358	68.7	39.6	59.0	28.3
	68.4	44.1	60.1	29.9

For the LINE-1 methylation data reconstructed from human remains at Conchopata, results of the pairwise Wilcoxon rank-sum tests are given in Table 3.7. We hypothesized that social outsiders at Conchopata (EA1 and EA20) would show differential methylation of LINE-1 compared to the intermediate elites (EA6, EA88, and EA205).

However, there are no statistically significant differences in any of the pairwise comparisons of LINE-1 methylation at Conchopata. The old woman at Conchopata (EA1) falls in the middle of the distribution of methylation values among intermediate elites at the site. She does not fall below the distribution of intermediate elites, as might be predicted based on existing studies of social inequalities in living humans (Zhang et al. 2011a, 2011b). We also predicted that the sacrificed woman who was born outside the community (EA20) would show differential methylation compared to those who were born in or near Conchopata. While the sacrificed woman does show the highest methylation of CpG position 2 of anyone at the site, the difference is not statistically significant.

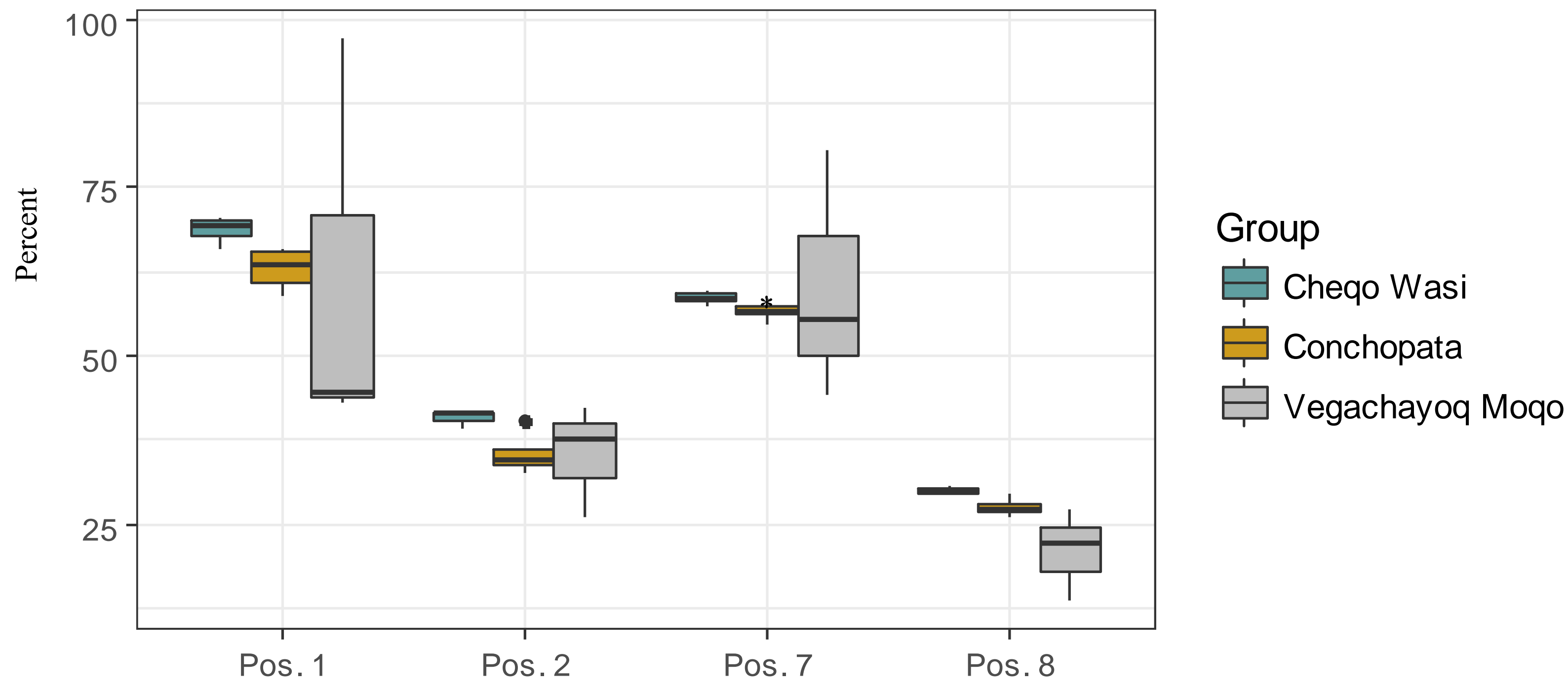
Table 3.7: Holm-Bonferroni corrected p-values from pairwise Wilcoxon rank-sum tests comparing percent methylation of LINE-1 CpG position 2 between individuals interred at Conchopata.

	EA1	EA6	EA20	EA88
EA6	0.98	-	-	-
EA20	0.43	0.43	-	-
EA88	0.18	0.98	0.70	-
EA205	0.98	0.98	0.70	0.98

We also compared LINE-1 methylation patterns across the three archaeological localities, to assess how methylation patterns might be changing over space and time across the Wari transition (Figure 3.11). Generally, elite individuals interred at Cheqo Wasi have the highest percent methylation across all four CpG sites and the least variability between individuals. Intermediate elites and non-elites interred at Conchopata are hypomethylated relative to elites at Cheqo Wasi, and there is slightly higher variability between individuals. People interred at the post-Wari site of Vegachayoq Moqo show the highest variability between individuals. One-way ANOVAs of the percent methylation values between

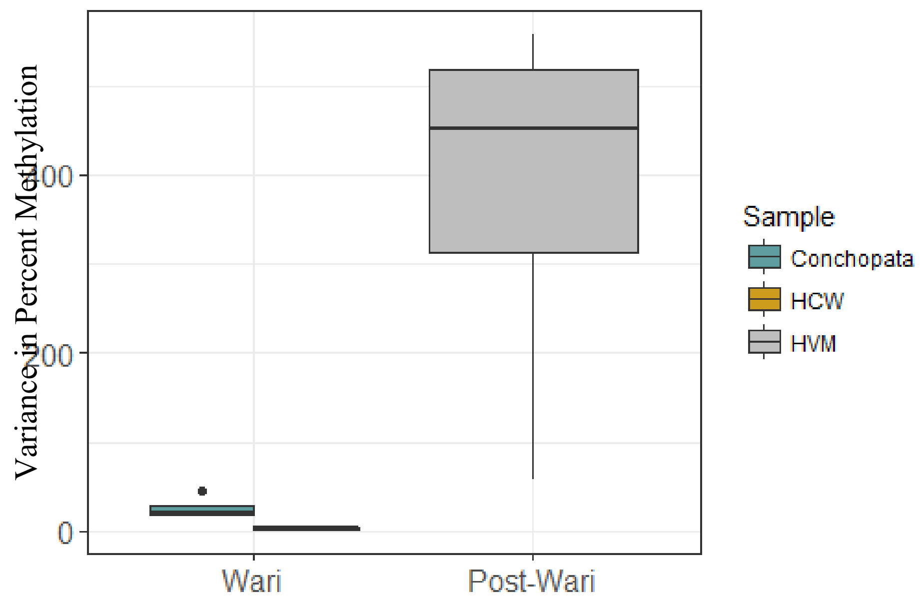
localities for each CpG position show that there is a statistically significant difference between localities at CpG position 8 ($p=0.037$).

Figure 3.11: LINE-1 methylation patterns across three Wari and post-Wari archaeological localities.



Finally, to test the hypothesis that variance in LINE-1 methylation would be greater between individuals living in post-Wari times relative to Wari times, we grouped the samples into Wari and post-Wari cohorts and calculated the variance in percent methylation values for each of the four CpG positions included in the LINE-1 analysis. We then performed a one-way ANOVA to test whether variance in percent methylation between individuals was greater after the decline of the Wari state. Our results show that variation in LINE-1 methylation during post-Wari times was significantly greater than during Wari times (Figure 3.12, $p=0.00072$, $\eta^2=0.70$). The variance in methylation between the two Wari era populations was also significantly different ($p=0.012$, $\eta^2=0.67$), but less so than the difference between Wari and post-Wari populations. Taken together, we observed statistically significant differences in methylation across the three archaeological periods, but the largest effect was observed when samples were grouped into Wari and post-Wari populations.

Figure 3.12: Variance in methylation between Wari and post-Wari times.



DISCUSSION

LINE-1 Methylation in Contemporary Human Tissues

Epigenetic marks such as cytosine methylation are shaped by a variety of social and environmental factors (Thayer and Kuzawa 2011, Thayer and Non 2016, Vinkers et al. 2016). Because cytosine methylation can be reconstructed from aDNA, we evaluated whether emerging techniques in paleoepigenetics could provide insights into how sociopolitical and ecological events shaped ancient populations. However, methylation patterns are known to vary from tissue to tissue (Christensen et al. 2009, Varley et al. 2013, Lokk et al. 2014). Therefore, we first conducted an analysis of LINE-1 methylation across a variety of tissues in living humans, including teeth, buccal cells, blood, and hair. Our results indicated that methylation patterns obtained from archaeologically-available tissues such as teeth are reliable indicators of LINE-1 methylation patterns in peripheral tissues such as buccal cells and whole blood. For the region of the LINE-1 promoter that we analyzed, CpG position was a far better predictor of methylation patterns than tissue source was. This result indicates that LINE-1 methylation patterns for this locus are influenced more significantly by the particular CpG being studied than by the tissue source, and that methylation levels at each of the four CpG positions analyzed are generally conserved across tissue types.

However, there was variation between individuals one and two in how predicative molar methylation patterns were for other tissues. While LINE-1 methylation patterns in molars were predicative of methylation patterns across all tissues that were tested for individual two, our preliminary results indicate that methylation patterns in molars were good predictors of methylation patterns only in buccal cells and blood for individual one, but not methylation patterns in hair. Because individuals one and two had different life

histories, it is possible that certain life events have differential effects on LINE-1 methylation across various cell types. Alternatively, it is also possible that once epigenetic patterns have been altered by an event, they may not be retained across different cell types in the same ways over time. Because CpG position 7 of the LINE-1 promoter has also been analyzed in previous studies using the same pyrosequencing assay, we can compare methylation levels observed in this study with those of larger populations. The population average for CpG position 7 reported in previous studies ranges between 62.03-62.55% (Ricceri et al. 2014). In this study, methylation at CpG position 7 for individual two (60.9%) is similar to the population average reported in previous studies, but individual one's methylation levels are substantially lower (55.5%). Our observation that individual one is hypomethylated compared to individual two is also consistent studies showing that decreased methylation of LINE-1 elements can result from social differences and disparities in lived experiences (Zhang et al. 2011a, 2011b). However, because our preliminary findings are limited by sample size and exposure groups, we are unable to determine what specific factors may be influencing these patterns. Therefore, further research will be necessary to determine whether the conservation of LINE-1 methylation patterns across tissues is effected by differences in lived experiences such as disparities in diet and exposure to violence. However, our preliminary results suggest that LINE-1 methylation patterns are highly correlated across molars, buccal cells, and blood for both individuals tested.

These results help to frame interpretations of LINE-1 methylation patterns reconstructed from the DNA of ancient populations. We found that LINE-1 methylation patterns in molars can serve as excellent proxies for methylation patterns in buccal cells and blood, suggesting that paleoepigenetic LINE-1 data from ancient teeth may be readily

compared with epigenetic analyses of buccal cells and blood in living humans. However, methylation patterns in hair showed more variable correlation with those of other tissues, and were consistently outliers compared with methylation levels in molars, buccal cells, and blood for both individuals tested. While hair is preserved in some archaeological contexts and has been shown to be an excellent source of endogenous aDNA (Rasmussen et al. 2010, Bengtsson et al. 2012), our results suggest that LINE-1 methylation patterns in hair may not always be a reliable proxy for LINE-1 methylation patterns in other tissues. Our preliminary findings also suggest that LINE-1 methylation patterns in hair may not track differences in life histories in the same way as other tissues, and may not be as readily comparable to epigenetic studies that have been based on buccal cells and blood in living humans. Because some lived experiences may unevenly alter methylation patterns that might otherwise be correlated across tissues, hair may not be an ideal source tissue for detecting the epigenetic effects of social and environmental change.

LINE-1 Methylation in Ancient Wari and Post-Wari Populations

While a small but growing number of studies have shown that it is possible to reconstruct methylation patterns from ancient teeth, bone, and hair (Gokhman et al. 2016), this study applied paleoepigenetic methods within an archaeological context to assess whether changes in cytosine methylation patterns trace sociopolitical and environmental changes in ancient societies. At the Wari-era site of Conchopata, we hypothesized that low-status, social outsiders would be differentially methylated compared to local intermediate elites at the site. Our results show that the sacrificed woman who was born outside Conchopata (EA20) showed higher LINE-1 methylation than everyone who was born in or near Conchopata. Though this result was not statistically significant, the different LINE-1 methylation levels of individual EA20 mirror other distinctions observed in her mortuary

treatment and strontium isotope ratios, which together provide many independent lines of evidence that this person was an outsider at Conchopata. Based on existing epigenetic studies of social inequalities and LINE-1 methylation, we would predict that the old woman with annular cranial modification and extensive signs of skeletal injury would be hypomethylated relative to intermediate elites at Conchopata. Contrary to this prediction, the woman with annular cranial modification fell within the range of methylation values that we observed among intermediate elites at the site. Overall, we found no statistically significant differences between social outsiders and intermediate elites at Conchopata. However, our analysis is limited by sample size, and the addition of more individuals in each exposure group may help to identify epigenetic differences in future studies.

By reconstructing methylation patterns from three archaeological localities across different phases of Wari society, our findings suggest that methylation patterns may track social and environmental differences over space and time in the ancient world. We hypothesized that global genomic methylation of LINE-1 would decrease from Wari to post-Wari times, as access to carbon-enriched foods diminished and exposure to violence worsened for many people after the decline of the Wari state. We also hypothesized that variability in percent methylation would increase as Wari social orders disintegrated, adversity became more pronounced, and vulnerability became more erratically structured in the population. Our results offer some support for these hypotheses, indicating that there are significant differences in LINE-1 methylation patterns between populations living during and after the Wari era. While not all post-Wari individuals are hypomethylated relative to Wari era people, the lowest methylation values in this study were found among post-Wari individuals interred at Vegachayoq Moqo. Notably, everyone from Vegachayoq Moqo is uniformly hypomethylated relative to people at Cheqo Wasi and Conchopata at

the 8th CpG position of LINE-1, and this difference was statistically significant. In addition, while people living through both height and the terminal phases of Wari society showed relatively low variability between individuals, people living at Vegachayoq Moqo after the decline of the Wari show far greater variability in levels of cytosine methylation.

Wari elites at Cheqo Wasi are generally hypermethylated relative to other populations tested, Wari intermediate elites and non-elites from Conchopata fall in the middle of the range, while some or all non-elite individuals (depending on the CpG position) from Vegachayoq Moqo are hypomethylated relative to populations during the Wari-era. These findings generally align with studies of LINE-1 methylation in living humans, which have shown that nutritional differences and social inequalities can lead to genomic hypomethylation of repetitive elements. Taken together, the differences in LINE-1 methylation patterns observed between archaeological localities broadly reflect what is known about the social and environmental conditions that existed between populations in the Wari and post-Wari eras. We also found that variance in percent methylation was significantly different between the three archaeological periods, but the greatest effect was observed when people were grouped into Wari and post-Wari populations. However, because time period in this study is variably conflated with archaeological locality, status, and dietary and social factors, it is difficult to identify specific underlying causes for these patterns.

While our results suggest that LINE-1 methylation patterns track ancient social and environmental changes, our findings should be interpreted with caution for several reasons. First, aspects of the archaeological context present certain limitations. Given the relatively small number of samples with preserved aDNA in this study, our sample size is limited. In addition, while the three archaeological sites studied here provide important glimpses of

different lifeways in the Wari heartland, they cannot be taken to be representative of Wari society as a whole, or of the broader political and environmental conditions that existed across the Andes in the MH and LIP. For example, while the decline of the Wari seems to have greatly affected the heartland sites represented in this study, these events sometimes had less dramatic effects among the more distant, coastal communities that had once been part of the Wari political sphere. Thus, the localities included in this study provide a snapshot of differences in people's lived experiences in the Wari heartland, but these events are separated by time and geography in such a way that they cannot simply be viewed as part of a single overarching or continuous narrative. However, it is clear that the patterns, contexts, exposures and even the meaning of violence varied substantially over space and time across the Wari transition, and that differences in access to carbon-enriched foods was also affected by the decline of the Wari state for the populations included in this study. Finally, while it would be ideal to have samples from a single site to assess how cytosine methylation patterns change over time, these samples are not yet available for the Wari.

Another limitation of our study may stem from the effects of post-mortem deamination of cytosines, which we have argued elsewhere may 1) artificially reduce estimates of cytosine methylation in aDNA when using BS-seq and 2) lead to variability in independent measures of cytosine methylation (Smith et al. 2015). In this study, it is relatively unclear to what extent cytosine deamination may be influencing our results. Because the rate of cytosine deamination is an exponential process, our samples are likely to be affected by relatively similar rates of DNA degradation and we would not expect variability in the data to be shaped by differences in deamination rates between archaeological sites. In addition, we included controls for DNA degradation by running additional rounds of statistical tests that excluded samples with high variance between

independent replicate measures of methylation. Our findings remained consistent following these statistical controls. Next, the patterns of variability do not appear to be time dependent, as would be expected if DNA degradation were shaping our results. Samples from Vegachayoq Moqo were the youngest in this study, and had a greater proportion of samples with preserved DNA (40%) than the oldest samples from Conchopata (36%). In spite of this, the younger samples from Vegachayoq Moqo show the greatest variability in methylation between individuals of any site, while the older samples from Cheqo Wasi and Conchopata showed relatively little variability in methylation between individuals. In addition, mtDNA results from individuals interred at Vegachayoq Moqo indicate that these individuals had the best preserved aDNA among all the localities included in this study, providing further evidence that the variability in LINE-1 methylation we observed at this site does not stem from DNA degradation. Finally, the ancient methylation data incorporated into the most conservative statistical tests were no more variable between independent replicate measures of the same individual than what was observed among the two contemporary samples.

Finally, while LINE-1 methylation has often been used as a measure of global genomic methylation, and has been shown to be shaped by social and environmental differences, the biological significance of LINE-1 methylation remains somewhat unclear. LINE-1 methylation represents a gross assessment of methylation across the genome, and lacks specific functional outcomes. In addition, changes in the methylation of repetitive elements can be shaped by a variety of different factors, so methylation of these loci lack a single biological meaning, and it is often difficult to distinguish between multiple potential influences on LINE-1 methylation. Given what we know from previous bioarchaeological analyses of the Wari and post-Wari sites included in this study, it is likely

that multiple factors are operating simultaneously on these populations to shape the patterns of global genomic methylation observed here. Multiple social and environmental inputs on this locus also open the possibility of compensatory processes, in which one set of lived experiences decreases the methylation of these loci while others may increase methylation. This could help explain why some individuals do not fit within the predictions of our hypotheses, such as EA1. While she showed signs of intense trauma, there is little evidence that her diet differed substantially from the intermediate elites at Conchopata.

CONCLUSION

In this study, we have provided the first evidence that methylation patterns may mirror social and environmental change in the ancient world. While it is difficult to identify the specific factors shaping these patterns, ancient methylation levels generally trace the decline of the Wari civilization in the Wari heartland sites included in this study. Our findings provide support for the feasibility of future research on the epigenetic effects of ancient lifeways. While we focused on global genomic methylation of LINE-1 elements, future paleoepigenetic studies of single-copy loci that are known to be affected by diet, disease, trauma, or other factors could shed further light on lived experiences in the ancient world. Given this possibility, paleoepigenetics could provide additional methodological tools for testing paleoanthropological and archaeological hypotheses. While the field of epigenetics is providing new insights into the dynamism of the genome in response to a variety of social and environmental events in living humans, applying these findings within a paleoepigenetic context may provide a way to assess epigenetic shifts over many centuries or even millennia of social and environmental change. Thus, paleoepigenetics may provide important new tools for assessing how large scale social, political, and environmental changes can shape human biology.

REFERENCES

Arkush E and Stanish C. 2005. Interpreting Conflict in the Ancient Andes. Curr Anthropol 46:3–28.

Arkush E and Tung T. 2013. Patterns of War in the Andes from the Archaic to the Late Horizon: Insights from Settlement Patterns and Cranial Trauma. J Archaeol Res 21:307–369.

Bengtsson CF et al. 2012. DNA from keratinous tissue. Part I: hair and nail. Ann Anat 194:17–25.

Bird BW et al. 2011. A 2,300-year-long annually resolved record of the South American summer monsoon from the Peruvian Andes. PNAS 108(21): 8583–8588.

Bolnick DA et al. 2012. Nondestructive sampling of human skeletal remains yields ancient nuclear and mitochondrial DNA. Am J Phys Anthropol 147:293–300.

Bragayrac E. 1991. Archaeological excavations in the Vegachayoq Moqo Sector of Huari. In: Isbell WH, McEwan GF, editors. Huari Administrative Structure: Prehistoric Monumental Architecture and State Government (Dumbarton Oaks Pre-Columbian Conference Proceedings). Washington, D.C.: Dumbarton Oaks Research Library and Collection. p 71–80.

Briggs AW et al. 2010. Removal of deaminated cytosines and detection of in vivo methylation in ancient DNA. Nucleic Acids Res 38:1–12.

Burger RL et al. 2016. Obsidian Procurement and Cosmopolitanism at the Middle Horizon Settlement of Conchopata, Peru. Andean Past 12:22–44.

Campos PF et al. 2012. DNA in ancient bone - where is it located and how should we extract it? Ann Anat 194:7–16.

Cantone I and Fisher AG. 2013. Epigenetic programming and reprogramming during development. Nat Struct Mol Biol 20:282–289.

Christensen BC et al. 2009. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CPG island context. PLoS Genet 5:e1000602.

Covey RA. 2008. Multiregional perspectives on the archaeology of the Andes during the Late Intermediate Period (c. A.D. 1000-1400). J Archaeol Res 16:287–338.

Dabney J et al. 2013. Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. Proc Natl Acad Sci 110:15758–15763.

Feil R and Fraga MF. 2012. Epigenetics and the environment: emerging patterns and implications. Nat Rev Genet 13:97–109.

Finucane B et al. 2006. Human and animal diet at Conchopata, Peru: stable isotope evidence for maize agriculture and animal management practices during the Middle Horizon. J Archaeol Sci 33:1766–1776.

Gilbert MTP et al. 2005. Assessing ancient DNA studies. Trends Ecol Evol 20:541–4.

Gokhman D et al. 2016. Epigenetics: It's Getting Old. Past Meets Future in Paleoepigenetics. Trends Ecol Evol 31:290–300.

Gokhman D et al. 2014. Reconstructing the DNA Methylation Maps of the Neandertal and the Denisovan. Science 344(80):523–528.

Grunau C et al. 2001. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. Nucleic Acids Res 29:E65–5.

Hanghøj K et al. 2016. Fast, accurate and automatic ancient nucleosome and methylation maps with epiPALEOMIX. Mol Biol Evol.

Holm S. 1979. A Simple Sequentially Rejective Multiple Test Procedure. Scand J Stat 6:65–70.

Isbell WH and McEwan GF, eds. 1991. Huari Administrative Structure: Prehistoric Monumental Architecture and State Government. Washington D.C.: Dumbarton Oaks Research Library and Collections.

Isbell WH. 2004. Society for American Archaeology Mortuary Preferences : A Wari Culture Case Study from Middle Horizon Peru. Soc Am Archaeol 15:3–32.

Jónsson H et al. 2013. MapDamage2.0: Fast approximate Bayesian estimates of ancient DNA damage parameters. Bioinformatics 29:1682–1684.

Kaestle FA and Horsburgh KA. 2002. Ancient DNA in anthropology: Methods, applications, and ethics. Am J Phys Anthropol 119:92–130.

Kemp BM et al. 2009. Genetic continuity after the collapse of the Wari empire: Mitochondrial DNA profiles from Wari and post-Wari populations in the ancient Andes. Am J Phys Anthropol 140:80–91.

Llamas B, Holland ML, Chen K, Cropley JE, Cooper A, Suter CM. 2012. High-resolution analysis of cytosine methylation in ancient DNA. PLoS One [Internet] 7:e30226.

Lokk K et al. 2014. DNA methylome profiling of human tissues identifies global and tissue-specific methylation patterns. Genome Biol 15:r54.

Orlando L, Willerslev E. 2014. An epigenetic window into the past? Science 345(80):511–512.

Paabo S et al. 2004. Genetic Analyses from Ancient DNA. Annu Rev Genet 38:645–679.

Pedersen JS et al. 2014. Genome-wide nucleosome map and cytosine methylation levels of an ancient human genome. Genome Res 24:454–466.

Portela A and Esteller M. 2010. Epigenetic modifications and human disease. Nat Biotechnol 28:1057–1068.

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rasmussen M et al. 2010. Ancient human genome sequence of an extinct Palaeo-Eskimo. Nature 463:757–762.

Ricceri F et al. 2014. Seasonality modifies methylation profiles in healthy people. PLoS One 9:e106846.

Rohland N and Hofreiter M. 2007. Ancient DNA extraction from bones and teeth. Nat Protoc 2:1756–1762.

Schreiber KJ. 1991. Association Between Roads and Polities: Evidence for Wari Roads in Peru. In: Trombold CD, editor. Ancient Road Networks and Settlement Hierarchies in the New World. Cambridge: Cambridge University Press. Pp 243-52.

Schreiber KJ. 1992. Wari Imperialism in Middle Horizon Peru. Ann Arbor: Museum of Anthropology, University of Michigan.

Seguin-Orlando A et al. 2015. Pros and cons of methylation-based enrichment methods for ancient DNA. Sci Rep 5:11826.

Shapiro B and Hofreiter M (eds). 2012. Ancient DNA. Methods and Protocols. Methods in Molecular Biology vol 840. Springer, New York.

Smith O et al. 2014. Genomic methylation patterns in archaeological barley show de-methylation as a time-dependent diagenetic process. Sci Rep 4:5559.

Smith RWA et al. 2015. Detection of cytosine methylation in ancient DNA from five native American populations using bisulfite sequencing. PLoS One 10:1–23.

Smith RWA et al. 2016. Evaluating Deamination-based Approaches for Inferring DNA Methylation: Insights from Two Ancient Genomes. AJPA 159:296-296.

Tanaka K and Okamoto A. 2007. Degradation of DNA by bisulfite treatment. Bioorganic Med Chem Lett 17:1912–1915.

Thayer ZM, Kuzawa CW. 2011. Biological memories of past environments: Epigenetic pathways to health disparities. Epigenetics 6:798–803.

Thayer ZM, Non AL. 2015. Anthropology Meets Epigenetics: Current and Future Directions. Am Anthropol 117:722–735.

Thompson LG et al. 2013. Annually resolved ice core records of tropical climate variability over the past ~1800 years. Science 340(6161): 945–950.

Tung T. 2012. Violence, Ritual, and the Wari Empire: A Social Bioarchaeology of Imperialism in the Ancient Andes. Gainesville: University of Florida Press.

Tung TA and Cook AG. 2006. Intermediate-Elite Agency in the Wari Empire. In: Elson CM, Covey RA, editors. Intermediate Elites in Pre-Columbian States and Empires. Tuscon: University of Arizona Press. p 68–93.

Tung TA and Knudson KJ. 2008. Social Identities and Geographical Origins of Wari Trophy Heads from Conchopata, Peru. Curr Anthropol 49:915–925.

Tung TA et al. 2016. Patterns of Violence and Diet Among Children During a Time of Imperial Decline and Climate Change in the Ancient Peruvian Andes. In: VanDerwarker AM, Wilson GD, editors. The Archaeology of Food and Warfare: Food Insecurity in Prehistory. Springer. p 193–228.

Tung TA. 2008. Violence After Imperial Collapse: A Study of Cranial Trauma among Late Intermediate Period Burials from the Former Huari Capitol, Ayacucho, Peru. J Andean Archaeol 29:101–117.

Tung TA. 2014a. Making Warriors Making War: Violence and Militarism in the Wari Empire. In: Scherer A, Verano J, editors. Embattled Bodies, Embattled Places: War in Pre-Columbian Mesoamerica and the Andes. Washington, D.C.: Dumbarton Oaks. p 227–256.

Tung TA. 2014b. Violence against Women: Differential Treatment of Local and Foriegn Females in the Heartland of the Wari Empire, Peru. In: Martin DL, Harrod RP, Orulgd ERI, editors. The Bioarchaeology of Violence. Gainesville: University Press of Florida. p 180–198.

Varley KE et al. 2013. Dynamic DNA methylation across diverse human cell lines and tissues. Genome Res 23:555–67.

Vinkers CH et al. 2015. Traumatic stress and human DNA methylation: a critical review. Epigenomics 7:593–608.

Williams PR. 2002. Rethinking Disaster-induced Collapse in the Demise of the Andean Highland States: Wari and Tiwanaku. World Archaeology 33(3):361-74.

Willerslev E, Cooper A. 2005. Ancient DNA. Proc R Soc B Biol Sci 272:3–16.

Zhang FF et al. 2011a. Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. Epigenetics 6:623–629.

Zhang FF et al. 2011b. Dietary Patterns Are Associated with Levels of Global Genomic DNA Methylation in a Cancer-Free Population. J Nutr:1165–1171.

Ziller MJ et al. 2014. Charting a dynamic DNA methylation landscape of the human genome. Nature 500:477–481.

COMPREHENSIVE REFERENCES

Alaimo S and Hekman S. 2008. Material Feminisms. Bloomington: Indiana University Press.

Andersen C. 2014. Metis: Race, Recognition, and the Struggle for Indigenous Peoplehood. Vancouver: UBC Press.

Arkush E and Stanish C. 2005. Interpreting Conflict in the Ancient Andes. Curr Anthropol 46:3–28.

Arkush E and Tung T. 2013. Patterns of War in the Andes from the Archaic to the Late Horizon: Insights from Settlement Patterns and Cranial Trauma. J Archaeol Res 21:307–369.

Barad K. 2007. Meeting the Universe Halfway: Quantum Physics and the Entanglement of Matter and Meaning. Durham: Duke University Press.

Bengtsson CF et al. 2012. DNA from keratinous tissue. Part I: hair and nail. Ann Anat 194:17–25.

Bird BW et al. 2011. A 2,300-year-long annually resolved record of the South American summer monsoon from the Peruvian Andes. PNAS 108(21): 8583–8588.

Bolnick DA et al. 2006. Asymmetric male and female genetic histories among Native Americans from Eastern North America. Mol Biol Evol 23:2161–74.

Bolnick DA et al. 2012. Nondestructive sampling of human skeletal remains yields ancient nuclear and mitochondrial DNA. Am J Phys Anthropol 147:293–300.

Bolnick DA et al. 2016. Native American Genomics and Population Histories. Annu Rev Anthropol:1–22.

Bolnick DA and Smith DG. 2003. Unexpected Patterns of Mitochondrial DNA Variation among Native Americans from the Southeastern United States. Am J Phys Anthropol 122:336–354.

Bragayrac E. 1991. Archaeological excavations in the Vegachayoq Moqo Sector of Huari. In: Isbell WH, McEwan GF, editors. Huari Administrative Structure: Prehistoric Monumental Architecture and State Government (Dumbarton Oaks Pre-Columbian Conference Proceedings). Washington, D.C.: Dumbarton Oaks Research Library and Collection. p 71–80.

Briggs AW et al. 2010. Removal of deaminated cytosines and detection of in vivo methylation in ancient DNA. Nucleic Acids Res 38:1–12.

Burger RL et al. 2016. Obsidian Procurement and Cosmopolitanism at the Middle Horizon Settlement of Conchopata, Peru. Andean Past 12:22–44.

Campos PF et al. 2012. DNA in ancient bone - where is it located and how should we extract it? Ann Anat 194:7–16.

Cantone I and Fisher AG. 2013. Epigenetic programming and reprogramming during development. Nat Struct Mol Biol 20:282–289.

Christensen BC et al. 2009. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CPG island context. PLoS Genet 5:e1000602.

Cooper A and Poinar HN. 2000. Ancient DNA: do it right or not at all. Science 289:1139.

Covey RA. 2008. Multiregional perspectives on the archaeology of the Andes during the Late Intermediate Period (c. A.D. 1000-1400). J Archaeol Res 16:287–338.

Dabney J et al. 2013. Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. Proc Natl Acad Sci 110:15758–15763.

Excoffier L and Lischer HEL. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564–567.

Feil R and Fraga MF. 2012. Epigenetics and the environment: emerging patterns and implications. Nat Rev Genet 13:97–109.

Finucane B et al. 2006. Human and animal diet at Conchopata, Peru: stable isotope evidence for maize agriculture and animal management practices during the Middle Horizon. J Archaeol Sci 33:1766–1776.

Fu Q, et al. 2013. A revised timescale for human evolution based on ancient mitochondrial genomes. Curr Biol 23:553–559.

Gilbert MTP et al. 2005. Assessing ancient DNA studies. Trends Ecol Evol 20:541–4.

Gokhman D et al. 2014. Reconstructing the DNA Methylation Maps of the Neandertal and the Denisovan. Science 344(80):523–528.

Gokhman D et al. 2016. Epigenetics: It's Getting Old. Past Meets Future in Paleoepigenetics. Trends Ecol Evol 31:290–300.

Grunau C et al. 2001. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. Nucleic Acids Res 29:E65–5.

Hanghøj K et al. 2016. Fast, accurate and automatic ancient nucleosome and methylation maps with epiPALEOMIX. Mol Biol Evol.

Haraway D. 1992. The Promises of Monsters. Abingdon: Routledge.

Holm S. 1979. A Simple Sequentially Rejective Multiple Test Procedure. Scand J Stat 6:65–70.

Hughes CE. Genetic structure of First Nation communities in the Pacific Northwest (unpublished).

Isbell WH. 2004. Society for American Archaeology Mortuary Preferences : A Wari Culture Case Study from Middle Horizon Peru. Soc Am Archaeol 15:3–32.

Isbell WH and McEwan GF, eds. 1991. Huari Administrative Structure: Prehistoric Monumental Architecture and State Government. Washington D.C.: Dumbarton Oaks Research Library and Collections.

Jónsson H et al. 2013. MapDamage2.0: Fast approximate Bayesian estimates of ancient DNA damage parameters. Bioinformatics 29:1682–1684.

Kaestle F. 1998. Molecular evidence for prehistoric native American population movement: the numic expansion. (Doctoral Dissertation).

Kaestle F and Horsburgh KA. 2002. Ancient DNA in anthropology: Methods, applications, and ethics. Am J Phys Anthropol 119:92–130.

Kemp BM et al. 2009. Genetic continuity after the collapse of the Wari empire: Mitochondrial DNA profiles from Wari and post-Wari populations in the ancient Andes. Am J Phys Anthropol 140:80–91.

Kemp BM and Schurr TG. 2010. Ancient and Modern Genetic Variation in the Americas. In: Human Variation in the Americas p 12–50.

Kolopenuk J. 2014. Becoming Native American: Facializing Indigeneity in Canada through Genetic Signification and Subjection. Annual Meeting of the Native American and Indigenous Studies Association. Austin, Texas.

Lin X, et al. 2016. Mass Spectrometry Based Ultrasensitive DNA Methylation Profiling Using Target Fragmentation Assay. Anal Chem 88(2): 1083–1087.

Lindo AJ, et al. 2016. Demographic and immune-based selection shifts before and after European contact inferred from 50 ancient and modern exomes from the Northwest Coast of North America. bioRxiv:1–16.

Llamas B, et al. 2012. High-resolution analysis of cytosine methylation in ancient DNA. PLoS One 7:e30226.

Lokk K et al. 2014. DNA methylome profiling of human tissues identifies global and tissue-specific methylation patterns. Genome Biol 15:r54.

Lorenz JG and Smith D. 1997. Distribution of Sequence Variation in the mtDNA Control Region of Native North Americans. Hum Biol 69:749–776.

Malhi RS et al. 2001. Distribution of mitochondrial DNA lineages among Native American tribes of Northeastern North America. Hum Biol an Int Rec Res 73:17–55.

Morgensen S. 2011. Spaces Between Us: Queer Settler Colonialism and Indigenous Decolonization. Minneapolis: University of Minnesota Press.

Mortimer-Sandilands C and Erickson B, eds. 2010. Queer Ecologies: Sex, Nature, Politics, Desire. Bloomington: Indiana University Press.

O'Rourke DH and Raff JA. 2010. The human genetic history of the Americas: the final frontier. Curr Biol 20:R202–7.

Orlando L, Willerslev E. 2014. An epigenetic window into the past? Science (80)345:511–512.

Paabo S et al. 2004. Genetic Analyses from Ancient DNA. Annu Rev Genet 38:645–679.

Patil V, et al. 2014. The evidence for functional non-CpG methylation in mammalian cells. Epigenetics 9:823–828.

Pedersen JS et al. 2014. Genome-wide nucleosome map and cytosine methylation levels of an ancient human genome. Genome Res 24:454–466.

Portela A and Esteller M. 2010. Epigenetic modifications and human disease. Nat Biotechnol 28:1057–1068.

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Raff JA et al. 2011. Ancient DNA perspectives on American colonization and population history. Am J Phys Anthropol 146:503–14.

Raghavan M et al. 2015. Genomic evidence for the Pleistocene and recent population history of Native Americans. Science 349(6250):1-11.

Rasmussen M et al. 2010. Ancient human genome sequence of an extinct Palaeo-Eskimo. Nature 463:757–762.

Rasmussen M et al. 2015. The ancestry and affiliations of Kennewick Man. Nature 523:455-458

Reich D et al. 2012. Reconstructing Native American population history. Nature488:370–4.

Renaud G, et al. 2015. Schmutzi: estimation of contamination and endogenous mitochondrial consensus calling for ancient DNA. Genome Biol 16:1–18.

Ricceri F et al. 2014. Seasonality modifies methylation profiles in healthy people. PLoS One 9:e106846.

Rohland N and Hofreiter M. 2007. Ancient DNA extraction from bones and teeth. Nat Protoc 2:1756–1762.

Rohland N, et al. 2014. Partial UDG-treatment for screening of ancient DNA. Philos Trans R Soc Biol:1–38.

Schreiber KJ. 1991. Association Between Roads and Polities: Evidence for Wari Roads in Peru. In: Trombold CD, editor. Ancient Road Networks and Settlement Hierarchies in the New World. Cambridge: Cambridge University Press. Pp 243-52.

Schreiber KJ. 1992. Wari Imperialism in Middle Horizon Peru. Ann Arbor: Museum of Anthropology, University of Michigan.

Schroeder KB et al. 2011. Biological Continuity in the Central Valley: Evidence from Ancient and Modern Mitochondrial DNA. J Calif Gt Basin Anthropol 31:37–56.

Seguin-Orlando A et al. 2015. Pros and cons of methylation-based enrichment methods for ancient DNA. Sci Rep 5:11826.

Shapiro B and Hofreiter M (eds). 2012. Ancient DNA. Methods and Protocols. Methods in Molecular Biology vol 840. Springer, New York.

Smith DG et al. 1999. Distribution of mtDNA haplogroup X among Native North Americans. Am J Phys Anthropol 110:271–84.

Smith O et al. 2014. Genomic methylation patterns in archaeological barley show de-methylation as a time-dependent diagenetic process. Sci Rep 4:5559.

Smith RWA et al. 2015. Detection of cytosine methylation in ancient DNA from five native American populations using bisulfite sequencing. PLoS One 10:1–23.

Smith RWA et al. 2016. Evaluating Deamination-based Approaches for Inferring DNA Methylation: Insights from Two Ancient Genomes. AJPA 159:296-296.

Sullivan et al. 1993. A Rapid and Quantitative DNA Sex Test – Fluorescence-Based PCR Analysis of X and Y Homologous Gene Amelogenin. Biotechniques 15(4):636-8

TallBear K. 2013. Native American DNA: Tribal Belonging and the False Promise of Genetic Science. Minneapolis: University of Minnesota Press.

Tamm E et al. 2007. Beringian standstill and spread of Native American founders. PLoS One 2:e829.

Tanaka K and Okamoto A. 2007. Degradation of DNA by bisulfite treatment. Bioorganic Med Chem Lett 17:1912–1915.

Thayer ZM and Kuzawa CW. 2011. Biological memories of past environments: Epigenetic pathways to health disparities. Epigenetics 6:798–803.

Thayer ZM and Non AL. 2015. Anthropology Meets Epigenetics: Current and Future Directions. Am Anthropol 117:722–735.

Thompson LG et al. 2013. Annually resolved ice core records of tropical climate variability over the past ~1800 years. Science 340(6161): 945–950.

Tollefsbol T. 2014. Transgenerational Epigenetics. Cambridge: Academic Press.

Tung TA. 2008. Violence After Imperial Collapse: A Study of Cranial Trauma among Late Intermediate Period Burials from the Former Huari Capitol, Ayacucho, Peru. J Andean Archaeol 29:101–117.

Tung TA. 2012. Violence, Ritual, and the Wari Empire: A Social Bioarchaeology of Imperialism in the Ancient Andes. Gainesville: University of Florida Press.

Tung TA. 2014a. Making Warriors Making War: Violence and Militarism in the Wari Empire. In: Scherer A, Verano J, editors. Embattled Bodies, Embattled Places: War in Pre-Columbian Mesoamerica and the Andes. Washington, D.C.: Dumbarton Oaks. p 227–256.

Tung TA. 2014b. Violence against Women: Differential Treatment of Local and Foriegn Females in the Heartland of the Wari Empire, Peru. In: Martin DL, Harrod RP, Orulgd ERI, editors. The Bioarchaeology of Violence. Gainesville: University Press of Florida. p 180–198.

Tung TA et al. 2016. Patterns of Violence and Diet Among Children During a Time of Imperial Decline and Climate Change in the Ancient Peruvian Andes. In: VanDerwarker AM, Wilson GD, editors. The Archaeology of Food and Warfare: Food Insecurity in Prehistory. Springer. p 193–228.

Tung TA and Cook AG. 2006. Intermediate-Elite Agency in the Wari Empire. In: Elson CM, Covey RA, editors. Intermediate Elites in Pre-Columbian States and Empires. Tuscon: University of Arizona Press. p 68–93.

Tung TA and Knudson KJ. 2008. Social Identities and Geographical Origins of Wari Trophy Heads from Conchopata, Peru. Curr Anthropol 49:915–925.

Varley KE et al. 2013. Dynamic DNA methylation across diverse human cell lines and tissues. Genome Res 23:555–67.

Vinkers CH et al. 2015. Traumatic stress and human DNA methylation: a critical review. Epigenomics 7:593–608.

Willerslev E and Cooper A. 2005. Ancient DNA. Proc R Soc B Biol Sci 272:3–16.

Willey A. 2016. Undoing Monogamy: The Politics of Science and the Possibilities of Biology. Durham: Duke University Press.

Williams PR. 2002. Rethinking Disaster-induced Collapse in the Demise of the Andean Highland States: Wari and Tiwanaku. World Archaeology 33(3):361-74.

Zhang FF et al. 2011a. Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. Epigenetics 6:623–629.

Zhang FF et al. 2011b. Dietary Patterns Are Associated with Levels of Global Genomic DNA Methylation in a Cancer-Free Population. J Nutr:1165–1171.

Ziller MJ et al. 2014. Charting a dynamic DNA methylation landscape of the human genome. Nature 500:477–481.