More Arrows in the Ancient DNA Quiver: Use of Paleoepigenomes and Paleomicrobiomes to Investigate Animal Adaptation to Environment

Yichen Liu ,*,[1] Laura S. Weyrich,*,†,[1] and Bastien Llamas*,[1]

[1]Australian Centre for Ancient DNA, School of Biological Sciences, Environment Institute, University of Adelaide, Adelaide, South Australia, Australia

†Present affiliation: Department of Anthropology, The Pennsylvania State University, University Park, PA 16802, USA

***Corresponding authors:** E-mails: yichen.liu@adelaide.edu.au; lsw132@psu.edu; bastien.llamas@adelaide.edu.au.

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Abstract

Whether and how epigenetic mechanisms and the microbiome play a role in mammalian adaptation raised considerable attention and controversy, mainly because they have the potential to add new insights into the Modern Synthesis. Recent attempts to reconcile neo-Darwinism and neo-Lamarckism in a unified theory of molecular evolution give epigenetic mechanisms and microbiome a prominent role. However, supporting empirical data are still largely missing. Because experimental studies using extant animals can hardly be done over evolutionary timescales, we propose that advances in ancient DNA techniques provide a valid alternative. In this piece, we evaluate 1) the possible roles of epigenomes and microbiomes in animal adaptation, 2) advances in the retrieval of paleoepigenome and paleomicrobiome data using ancient DNA techniques, and 3) the plasticity of either and interactions between the epigenome and the microbiome, while emphasizing that it is essential to take both into account, as well as the underlying genetic factors that may confound the findings. We propose that advanced ancient DNA techniques should be applied to a wide range of past animals, so novel dynamics in animal evolution and adaption can be revealed.

Key words: **ancient DNA, paleomicrobiome, paleoepigenome, animal evolution.**

Introduction

How animals adapt to a changing environment is a fundamental question in evolutionary biology. Efforts have focused on the characterization of genetic processes, such as mutation, drift, and selection, that underlie animal adaptation (Gillespie 2004).

However, increasing evidence suggests that epigenetic modifications play a significant role in shaping animal phenotypes and response to environmental stimuli (Jirtle and Skinner 2007). In addition, the recently proposed concept of hologenome—which considers the genome of an organism as an aggregation of the genomes of both the host and its resident microorganisms (microbiota)—challenges the traditional genetic paradigms that focus on the sole host genome (Zilber-Rosenberg and Rosenberg 2008). Hence, both epigenetic modifications and microbiome alterations need to be considered when discussing the evolutionary history of a particular species.

Evolutionary studies largely rely on the comparison and interpretation of extant organisms' DNA sequences, which provide only indirect evidence of past events and usually require temporal calibration using known fossil or biogeographical evidence (Telford et al. 2015). Alternatively, genetic information can be directly obtained from archeological and paleontological remains using ancient DNA (aDNA) techniques (Rohland and Hofreiter 2007). In this case, dating methods can be applied to the sample, providing a reliable timestamp for the genetic data (Lorenzen et al. 2011; Cooper et al. 2015). Furthermore, methylomes (the methylation profile of a genome) and microbiomes (the totality of microbial genomes within a niche) have been recently recovered from ancient human remains (Adler et al. 2013; Gokhman et al. 2014; Pedersen et al. 2014; Hanghøj et al. 2016), which paves the way for the study of past interactions between animals and environments using epigenetics and microbiome.

This review aims to discuss the possible roles of the epigenome and microbiome in animal adaptation to rapidly changing environments. We further propose that the implementation of aDNA techniques to retrieve paleoepigenomes and paleomicrobiomes from a wide range of ancient animals has a great potential to track novel dynamic processes underlying animal adaptation over evolutionary timescales.

Challenges and Opportunities of Using aDNA in Studying Animal Evolution

aDNA is DNA retrieved from subfossil biological specimens (i.e., not yet fossilized). aDNA sequencing is a powerful way to recover ancient genomic information. However, aDNA is subject to postmortem decay and contamination, which can lead to biases and misinterpretation of paleogenetic

data (Cooper and Poinar 2000; Hofreiter et al. 2001). Even under favorable circumstances (e.g., permafrost), DNA continually degrades with time (Hofreiter et al. 2001; Dabney et al. 2013). Consequently, the proportion of endogenous aDNA retrieved from a given sample is typically very low; the DNA molecules are also highly fragmented, with an average fragment length typically <100 bp (Sawyer et al. 2012). DNA damage is also characterized by miscoding lesions (Hofreiter et al. 2001). In particular, cytosines (especially those in single-stranded overhangs that form the sticky ends of aDNA fragments) are susceptible to conversion to uracil via hydrolytic deamination (Briggs et al. 2010). After experimental amplification of DNA, cytosine deamination leads to cytosine-to-thymine (C-to-T) substitutions on the damaged DNA strand and guanine-to-adenine (G-to-A) substitutions on the complementary strand. Although such damage causes sequence errors in the final DNA sequence, the increase of C-to-T and G-to-A substitutions at fragment termini now serves as the gold standard to authenticate aDNA (Llamas, Valverde, et al. 2017).

Despite the technical challenges of aDNA research, the advent of high-throughput sequencing techniques enabled genome-wide analyses of ancient specimens, providing critical insights into the evolutionary history of several species (Miller et al. 2008; Shapiro and Hofreiter 2014; Soubrier et al. 2016). However, many questions regarding mammalian evolution remain unsolved. For example, one of the most intriguing areas of research is the mass megafauna extinctions that took place during the last glacial period (Late Pleistocene; 110–11.65 thousand years ago—ka) (Cooper et al. 2015). During this period, many megafaunal species went extinct, including the iconic woolly rhinoceros, mammoth, short-faced bear, short-faced kangaroo, and ground sloth. Whether humans, climate changes, or both caused the mass extinctions remains highly controversial (Lorenzen et al. 2011; Sandom et al. 2014; Cooper et al. 2015). The controversy partially stems from missing data, and additional information about behavior, diet, and physiology of ancient animals could offer important clues. Paleoepigenome and paleomicrobiome analyses provide alternative access to such information (Warinner, Speller, Collins, and Lewis 2015; Gokhman, Malul, et al. 2017). For instance, changes in the oral microbiota might indicate a change in diet, whereas methylation levels in genomic regulatory regions can relate to specific phenotypes; alternatively, a diseased state might be linked to pathogens identified within the microbiome or abnormal methylation profiles (De Filippo et al. 2010; Jones 2012; Cui et al. 2013).

More importantly, epigenomes and microbiomes respond to environmental cues and thus have the potential to capture fine-scale dynamics between animals and paleoenvironment, which might be obscured in genomic data (fig. 1). For example, extinct steppe bison experienced and survived the last ice age. They have been morphologically classified into over 50 species based on the past fossil record (McDonald 1981), whereas genetic data suggest that steppe bison are a single morphologically plastic species (Shapiro et al. 2004). Morphological change of past mammals has been suggested to be associated with the environment. For example, the reduction in body size of past bison and horse is correlated to climate fluctuations (Guthrie 2003; Martin et al. 2018), and ancient bison distributed within different geographical environments showed extensive cranial differences (Wilson 1996). Epigenetic modifications can affect animal morphology (e.g., coat color and tail morphology) without changing the underlying genomic sequences (Waterland and Jirtle 2003; Waterland et al. 2006). It is thus possible that the past bison morphological diversity is driven, at least in part, by epigenetic changes triggered by environmental factors. *Myotragus balearicus*, an extinct insular cave goat endemic to the Gymnesic Islands in the Mediterranean sea, appears to have adapted to feed on a plant (*Buxus balearica*) that is toxic to ruminants (Welker et al. 2014). Many mammalian herbivores employ their gut microbes to facilitate the degradation of harmful components in their diet (e.g., the desert woodrat [Kohl et al. 2014], koala [Shiffman et al. 2017], and the Japanese large wood mouse [Sasaki et al. 2005]). The gut microbiome of this goat might play a similar role by enabling tolerance to the toxic plant.

In these examples, aside from offering additional information about phenotypic alterations (e.g., modified body size and the ability to gain nutrients from otherwise toxic diets) and environmental cues (e.g., ice age and toxic vegetation), the epigenome and microbiome might serve as mechanisms that facilitated animal adaptation to the environment. However, this posit has not been fully explored. To investigate the evolutionary role of epigenetic modifications and microbiome variations, three major questions need to be addressed. First, how do epigenome and microbiome respond to environmental stimuli, and what are the phenotypic consequences? Second, can those epigenetic responses and microbiome changes be maintained over multiple generations and thus influence animal adaptation in the long term? Finally, how can research verify this hypothesis on an evolutionary timescale?

DNA Methylation Patterns as a Proxy to Infer Animal–Environment Interactions

Epigenetics refers to mechanisms that alter the expression of genes without modifying the underlying genetic sequence. This includes DNA methylation, histone modifications, nucleosome positioning, and noncoding RNAs (Holliday 2006). In this review, the discussion of epigenetic modification will focus on DNA methylation, as it is likely the most accessible epigenetic signal that can be recovered from aDNA (Llamas et al. 2012; Smith et al. 2015).

In mammalian genomes, DNA methylation is found almost exclusively in the context of CpG dinucleotides and typically occurs at the fifth carbon position of cytosines (Jones and Takai 2001). Taking humans as an example, it has been estimated that about 1% of all DNA bases are methylated (Kim et al. 2009; Ziller et al. 2013), and the global methylation level can vary depending on age, sex, and disease states (Tsang et al. 2016). However, locus-specific methylation can vary across differentiated cells and throughout the

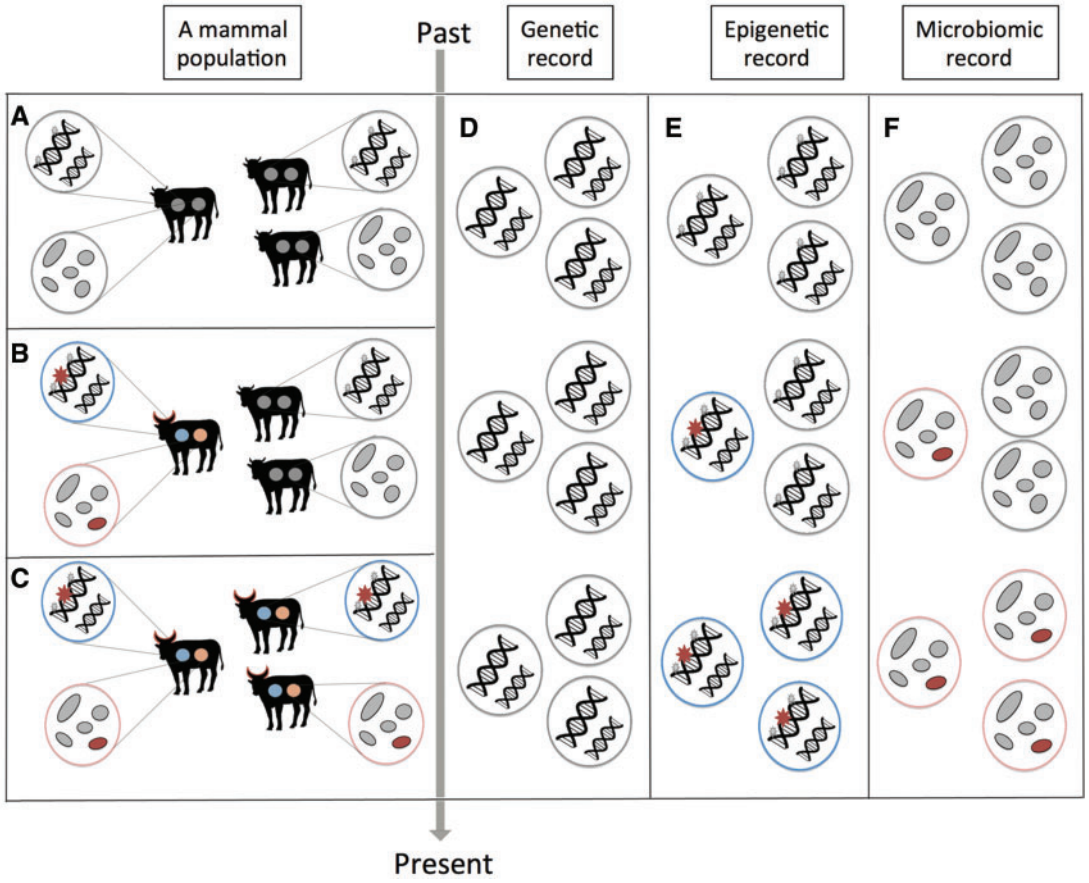

FIG. 1. Schematic figure of how the epigenome and microbiome can respond to environmental cues over time. (*A*–*C*) Environmentally induced epigenetic and microbiome alterations steadily increase within a mammal population. (*D*–*F*) Epigenetic and microbiome alterations are preserved within epigenetic and microbiome records, in the absence of genetic variation.

development of an organism (Smith and Meissner 2013). DNA methylation in promoter regions typically blocks the initiation of transcription, whereas methylation occurring within the gene body can alter gene expression, including stimulating transcript elongation and alternative splicing (Jones 2012). Due to their regulatory role, DNA methylation patterns are associated with a wide range of biological traits (Jones 2012).

Environmentally Induced Epigenetic Modifications

Animals are constantly exposed to the surrounding environment, which encompasses a wide range of beneficial or adverse factors that can exert physiological and psychological stresses and stimulate a series of adaptive responses (Koolhaas et al. 1999; Kent et al. 2014; Mellor 2015; Hay et al. 2016). DNA methylation can respond to various environmental cues, including early life nutrition, temperature, and many other factors (e.g., chemical compounds, hypoxia, and mental stress) (Shen et al. 2002; Bollati and Baccarelli 2010; Cao-Lei et al. 2014).

Early life nutrition is critical to fetal epigenetic programing and could give rise to persistent and systematic epigenetic alterations (Heijmans et al. 2008; Tobi et al. 2009; Cao-Lei et al. 2014). One possible explanation is that maternal nutrients are associated with the levels of methyl donors available as a biochemical substrate for DNA methylation (Niculescu and Zeisel 2002; Anderson et al. 2012). DNA methylation is enzymatically catalyzed by DNA methyltransferases, which transfer methyl groups from S-adenosylmethionine to the fifth carbon position of cytosines (Chiang et al. 1996; Fuso et al. 2005). The synthesis of S-adenosylmethionine requires the presence of methyl donors, such as folate, B6, B12, and some other dietary B vitamins (Fuso et al. 2005). In animals, the availability of methyl donors in the maternal diet can affect fetal epigenetic states, and consequently, affect the offspring's phenotype (Cooney et al. 2002; Waterland et al. 2006). For example, methyl donor supplementation of female mice before and during pregnancy increases DNA methylation at a metastable epiallele (i.e., the epigenetic state is determined during early development and can vary across individuals; once established, the epigenetic state is mitotically inherited), *axin fused* ($Axin^{Fu}$), which results in a decreased incidence of tail abnormality in their offspring (Waterland et al. 2006). Many epigenetically regulated genes play important roles in embryogenesis and development, and altered DNA methylation patterns caused by maternal nutrition can exert a life-long influence in mammals (Li et al. 1993; Okano et al. 1999).

Temperature fluctuations can pose challenges to animal adaptation to the environment and have the potential to

trigger alterations of DNA methylation in animals (Navarro-Martín et al. 2011; Cahill et al. 2013; Marsh and Pasqualone 2014; Parrott et al. 2014; Cooper et al. 2015). This temperature-related methylation has been best characterized in temperature-dependent sex determination in poikilothermic animals (Navarro-Martín et al. 2011; Parrott et al. 2014). Temperature-related DNA methylation and demethylation is less understood in mammals, but evidence suggests that temperature can prompt alterations in the mammalian methylome as well. For example, exposure to altered ambient temperature during adulthood is associated with the change of DNA methylation of multiple genes in blood cells (Bind et al. 2014, 2016). However, it is unclear if such alterations can happen to germ cells and be transmitted to offspring.

In nature, the alteration of nutrition availability and temperature is likely coupled with physiological and psychological stress. DNA methylation changes may arise as a direct response to environmental cues, or as an indirect response through stress. Prenatal exposure to environmental cues can trigger the alteration of DNA methylation of genes, including those with functions critical to respond to the environmental factors. Such environmentally induced methylation alterations have been observed in humans who experienced natural and unnatural disasters (e.g., Hunger Winter in 1944–1945 and 1998 Quebec ice storm) (Veenendaal et al. 2013; Cao-Lei et al. 2014). It is highly possible that epigenetic changes also occurred in animals when the environment changed drastically (e.g., during the Late Pleistocene, which was characterized by a series of dramatic cooling and warming events that altered the extent of ice sheets across the globe [Cooper et al. 2015]). However, these environmentally induced epigenetic marks must be passed down over multiple generations to ultimately be a substrate for natural selection.

Trans-Generational Effects of Epigenetic Modifications on Animal Adaptation

The plasticity and regulatory role of epigenetic modifications enable short-term exposure to environmental cues to be translated into phenotypic traits, and such epigenetic alterations (epimutations) have been shown to be maintained over multiple generations (Anway and Skinner 2006; Cropley et al. 2012). Trans-generational effects make epimutation-mediated natural selection theoretically possible: the environment triggers epimutations, which lead to phenotypic alterations that can be transmitted to offspring and subjected to natural selection. This process is best modeled in isogenic (i.e., all individuals are genetically identical) viable yellow agouti (A^{vy}) mice (Cropley et al. 2012). The A^{vy} allele is epigenetically regulated in mice and its expression impacts coat color. In this model, the environmentally induced epimutation was simulated by manipulating methyl donor supplementation in the mice diet, and natural selection was mimicked using selective breeding. Interestingly, the prevalence of epimutation-associated phenotypes was steadily increased in a population as long as the diet was supplemented with methyl donors (i.e., for five generations) (Cropley et al. 2012). As these mice are otherwise genetically identical, the change of coat color is only driven by an epigenetic response to an external factor and selective forces.

Note that the A^{vy} allele-related phenotype can be maintained only with dietary methyl donors (Cropley et al. 2012). However, heritable epigenetic modifications have been observed in pigs, rodents, and humans (Cropley et al. 2012; Wang et al. 2014; Skvortsova et al. 2018; Weyrich et al. 2018), where many genes (e.g., *IGF2R*, *Snrpn*, *Peg3*, *mest*, and *H19*) are epigenetically imprinted in gametes—that is, genes are expressed in a monoallelic and parent-of-origin manner (Dean et al. 1998). In the case of imprinting, and contrary to the A^{vy} allele-related phenotype that needs to be maintained with dietary supplements (Cropley et al. 2012), epialleles can persistently affect animal traits throughout their life even in the absence of obvious external stimuli. Environmental factors (including maternal nutrition) can influence imprinting during fetal development (Kappil et al. 2015). If the environment induced epigenetic adaptation, it would be invisible in the genetic record, as it does not entail genetic change. Nevertheless, the epigenetic modifications that occur in gametes or during early developmental stages can be mitotically passed down to different types of cells, and thus can be preserved in subfossil records (e.g., bones, teeth, and hair).

Methods and Progress in Ancient Epigenetic Research

Several methods are available to retrieve methylomes from modern samples, including bisulfite-based approaches and antibody-based enrichment (Harris et al. 2010). However, the characteristic fragmentation and damage of aDNA molecules pose serious limitations to these methods when analyzing ancient samples. In aDNA research, bisulfite conversion followed by targeted amplification is at best limited to a few methylated loci even using well-preserved samples, whereas antibody-based enrichment is biased toward large fragments and CpG-rich regions (Llamas et al. 2012; Seguin-Orlando et al. 2015; Smith et al. 2015). The inefficacy and bias of these methods have hindered the retrieval of methylome-wide data from ancient samples. However, the extensive damage that occurs in aDNA offers a proxy to evaluate cytosine methylation levels from ancient samples (Gokhman et al. 2014, 2019; Pedersen et al. 2014; Gokhman, Tamir, et al. 2017). The deamination of cytosines and methylated cytosines is the most frequently observed damage in aDNA (Hofreiter et al. 2001). This process converts cytosines to uracils (C-to-U) and methylated cytosines to thymines (5mC-to-T). After enzymatically removing the uracils using uracil-DNA-glycosylase (UDG), the remaining C-to-T substitutions in the sequencing data reflect the methylation level in a given region (Briggs et al. 2010). This method has helped to reconstruct some ancient and archaic human methylomes (Gokhman et al. 2014; Pedersen et al. 2014; Hanghøj et al. 2016).

However, the resolution of this method depends on the depth of coverage and is limited to a regional characterization of methylation. It seems experimental bisulfite conversion is still the gold standard for obtaining base-resolution methylomes (Leontiou et al. 2015). However, the biggest obstacle to apply bisulfite sequencing to ancient samples is the

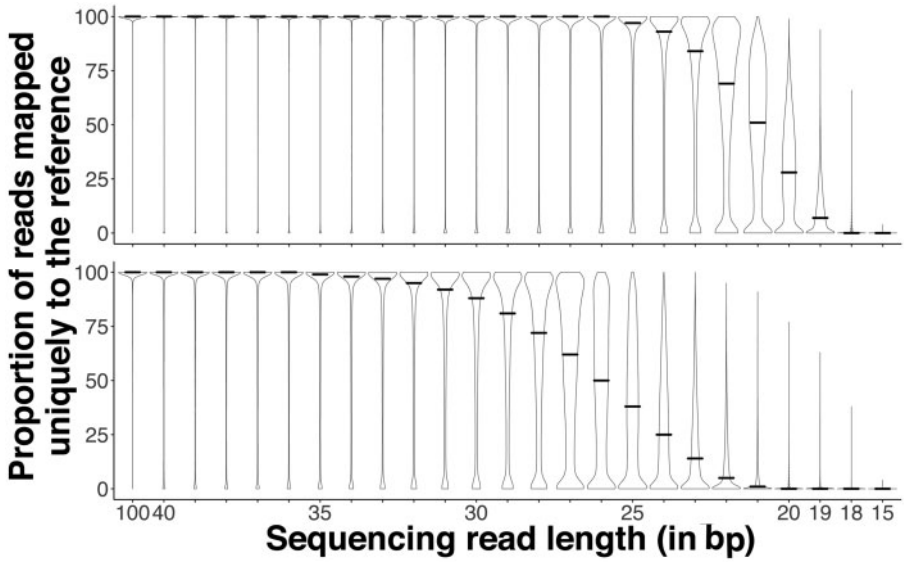

FIG. 2. The proportion of reads mapped uniquely to the reference genome. Top: the proportion of uniquely mapped ATCG-coded reads drops when the sequencing read length <26 bp. Bottom: the proportion of uniquely mapped ATG-coded reads drops when the sequencing read length <36 bp.

combination of short fragment length and reduced sequence complexity. Bisulfite converts cytosines to uracils and leaves methylated cytosines unchanged, making it possible to differentiate cytosines from methylated cytosines (Leontiou et al. 2015). Consequently, in most of the genome, cytosines are not methylated and will be displayed as thymines, which means that the original ATGC-coded DNA becomes an ATG-coded sequence. In aDNA, this means short fragments can no longer be mapped to the reference genome unambiguously. To illustrate this, we estimated the minimal lengths of ATGC-coded and ATG-coded sequences required for a unique mapping to a 2.6-Gb reference genome. Specifically, 20 subsets of reads (data available upon request) were extracted from the genome sequencing data of a wood bison mapped to the *Bos taurus* reference genome UMD 3.1.1. Each subset contains $n = 100{,}000$ uniquely mapped reads that were 150 bp in length. These reads were randomly fragmented in silico into varied sizes ranging from 15 to 100 bp ($n = 100{,}000$ for each size) and then mapped to UMD 3.1.1. The proportion of uniquely mapped reads was calculated. The mappability of ATG-coded short reads was evaluated the same way except all the C in the reads and UMD3.1.1 were converted to T in silico prior to mapping. We found that accurate mapping of a bison genome requires at least 26-bp-long DNA fragments, whereas a minimum of 36 bp are necessary after bisulfite conversion of cytosines (fig. 2). Given the fact that most aDNA fragments are very small, 10 bp can make a significant difference in terms of the amount of data that can be obtained from an individual sample.

This issue has been recently addressed by a bisulfite sequencing method optimized for aDNA (Llamas, Heiniger, et al. 2017) based on (Laird et al. 2004; Zhao et al. 2014). Specifically, a hairpin adaptor is ligated to one end of the DNA molecules before bisulfite conversion. The bisulfite treatment denatures the hairpin molecules but the plus and minus strands remain attached via the hairpin adaptor. Both strands are thus sequenced together using paired-end sequencing and, after folding the sequencing reads bioinformatically, it is possible to reconstruct the original DNA sequence and identify methylation. Because the original ATGC-coded DNA can be recovered, the method allows accurate mapping of short fragments and detection of methylation at a single-base resolution. Thus, this method can be applied to retrieve highly resolved ancient methylomes.

Ancient epigenetics research is still in its infancy and only a small number of ancient methylomes have been reconstructed. However, this small data set revealed intriguing findings. Altered DNA methylation profiles were detected from hominids such as Neanderthals and Denisovans (Gokhman et al. 2014, 2019; Gokhman, Tamir, et al. 2017). The identified alterations have been related to phenotypic variation, including limb, facial, and vocal tract morphology. These phenotypes are very likely to play critical roles in hominid evolution. For instance, the facial and vocal morphology can affect speech, which is considered unique to anatomically modern humans (Rauschecker and Scott 2009). Different hominin lineages also seem to have a distinctive set of methylation signatures (Gokhman, Tamir, et al. 2017; Gokhman et al. 2019), which might be a projection of corresponding epigenetic response to both the environment and underlying genetics. Additionally, although not necessarily heritable, many DNA methylation loci are environmentally responsive, which can offer information about past lifestyles and environments, as reviewed in Gokhman, Malul, et al. (2017). A striking example given by Gokhman et al. is the similar hypermethylation of EXD3, RBM46, and ZNF678 in Gambian hunter-gatherer children conceived during periods of caloric intake restriction (rainy season, or "hungry" season), and archaic humans such as Neandertals and Denisovans. It is, therefore, possible to hypothesize that archaic humans, who had a

hunter-gatherer lifestyle, experienced "hungry" seasons equivalent to those experienced by Gambian hunter-gatherers. With the new methods tailored for aDNA, both the quality and quantity of paleomethylome data are likely to increase rapidly.

Host–Microbiome Interactions and Their Implications in Environmental Adaptation

The microbiome is another indispensable part of animal biology that may play vital roles in animal adaptation. Trillions of microorganisms (microbiota) inhabit various body sites within animals, but the importance of these microbial communities and their genomic diversity (microbiome) was underestimated until recently (Huttenhower et al. 2012). Indeed, microbiota outnumber their host's cells (Sender et al. 2016). The vast numbers of microbes dynamically interact with their host in a complex and often beneficial manner: they can influence the maturation of host immunity, provide key nutrients, and affect host metabolism (Kinross et al. 2011; Huttenhower et al. 2012; Lloyd-Price et al. 2016). The disruption of microbiota and their functions is often associated with the development of many diseases (e.g., obesity, diabetes, mental health, skin disorders, and cancer) (Grice and Segre 2011; Devaraj et al. 2013; Schwabe and Jobin 2013; Hartstra et al. 2015). Because of their known relationships to animal health, gut and oral microbiomes are among the most extensively studied areas of microbiome research.

Different body sites are colonized by distinct microbiota, and the oral cavity harbors an especially diverse microbiota that is distinct from other body sites (Gill et al. 2006; Turnbaugh et al. 2007; He et al. 2015). The mouth of a healthy animal is typically home to over 200 microbial species (Dewhirst et al. 2010, 2012), which belong to Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Fusobacteria phyla (He et al. 2015). Although many microbes in the mouth are planktonic, other species readily form a biofilm on the surface of teeth and soft epithelial tissues. Several *Streptococcus* species are key, primary players in dental plaque formation, as they can adhere to tooth enamel and allow the secondary binding of other bacteria, which can dictate oral health outcomes (Dewhirst et al. 2010). Furthermore, the oral microbiota plays roles in systemic diseases, including cardiovascular disease, diabetes, and cancer (Nakano et al. 2009; Ahn et al. 2012; Hartstra et al. 2015; Shoemark and Allen 2015).

Like the mouth, the gastrointestinal tract harbors an extensively studied microbiota that is responsible for food digestion, nutrition absorption, and intestine functions (Turnbaugh et al. 2009; Arumugam et al. 2011; Kau et al. 2011). The gut microbiota can transform indigestible molecules into smaller and digestible nutrients, thereby increasing the nutrient bioaccessibility for the hosts (Kau et al. 2011). For example, host enzymes within the intestine cannot digest dietary fibers, whereas Bacteroidetes bacteria, part of the core gut microbiota, can transform fibers into physiologically active metabolites, such as short-chain fatty acids (Trompette et al. 2014). Gut microbiota can also synthesize essential vitamins (including B-group vitamins and vitamin K) and thus act as micronutrient suppliers (LeBlanc et al. 2013). Besides facilitating intestine function, gut microbiota are also involved in microbial detoxification and play a role in modulating brain development and behavior (Heijtz et al. 2011; Kohl et al. 2014, 2016).

Factors That Contribute to Microbiota Variation

The gut microbiota exhibits great flexibility and plasticity and is dynamically shaped by host and environment (Kinross et al. 2011; Spor et al. 2011). Although gut microbial communities vary according to host species, the predominant factor that influences the animal gut microbiota is diet. Herbivorous, carnivorous, and omnivorous animal gut microbiotas cluster into distinct groups (Ley et al. 2008), and different microbiota compositions can be found in same animal species with different dietary habits. Bacteriodetes dominate the gut microbiota of children whose diet has a high content of carbohydrates, fiber, and nonanimal proteins, whereas Firmicutes dominate the gut microbiota of children whose diet is rich in animal proteins, sugar, and starch (De Filippo et al. 2010). These dietary differences can also be sex based. In the Hadza hunter-gatherers, *Treponema* species capable of fiber digestion are increased in Hadza women, which likely results from an adaptation to the higher amount of tubers in their diet compared with men (Schnorr et al. 2014). In addition to diet, numerous other factors, including seasonality, habitat, and altitude, can also impact the microbiota in animals (Adak et al. 2013; Guan et al. 2017; Smits et al. 2017).

Although multiple factors can affect animal gut microbiota, these changes can potentially influence host metabolism, disease susceptibility, behavior, and consequently impact animal adaptation to the environment. The ability to synthesize various micronutrients and influence the host digestive efficiency might be vital for animals survival during rapid environmental changes and during times when food intake is insufficient and nutrient deficiencies occur (Allen et al. 2010). Dietary or environmentally induced adaptive changes in microbiota have been observed in mammals. For instance, some desert woodrats (*Neotoma lepida*) have a specialized gut microbiota for detoxification, allowing an adaptation to a toxic diet (Kohl et al. 2014), whereas giant pandas (*Ailuropoda melanoleuca*) harbor a gut microbiota with increased cellulose and lignin degradation activities for their adaptation to a bamboo-dominated diet (Zhu et al. 2011). In addition, during times of infectious disease stress, unique microbes may provide protection, or past exposure to related microorganisms may even provide immunity to some diseases (Kinross et al. 2011).

Trans-Generational Effects of Microbiota on Animal Adaptation

One of the most important foundations of coevolution and coadaptation between mammals and their microbiota is that parental (mostly maternal) microbiota can be passed down through vertical transmission to the offspring (Li et al. 2005; Cho and Blaser 2012). Direct contact between infants

and vaginal microbes during birth, as well as subsequent mother–infant interactions (e.g., breastfeeding and direct food sharing) can mediate the transmission of microbiota (Hyman et al. 2014). The transmission of microorganisms in later stages of life is less clear, although there is evidence suggesting it continues through life (Song et al. 2013).

In the long term, vertically transmitted microbes can contribute to animal adaptation independently of the genome. The giant panda is such an example. Genomic and morphological evidence strongly supports that the giant panda belongs to the Ursidae family, which is primarily composed of carnivore species. However, despite the fact that the genome of the giant panda contains genes encoding enzymes for meat digestion, the giant panda's diet is primarily herbivorous and consists almost exclusively of bamboo (Dierenfeld et al. 1982; Li et al. 2010). The discordance between phylogeny and feeding habit may be explained by its gut microbiota. Although the overall gut microbiome profile of giant panda is similar to that of a carnivore (Xue et al. 2015), functional analysis revealed the atypical presence of microbial genes encoding enzymes that participate in cellulose metabolism, which is likely a key element that enables a bamboo-centric diet (Zhu et al. 2011). Similarly, koalas rely on tailored gut microorganisms to aid in the detoxification of the plant material that they consume and mediate the transfer of these crucial microbes through coprophagy (Osawa et al. 1993). In either case, the direct transfer of microorganisms is essential for specific adaptations to a given environment or diet. Several bacterial strains from Bacteroidaceae and Bifidobacteriaceae show strong evidence of vertical transmission (Jost et al. 2014; Milani et al. 2015). A proportion of vertically transmitted bacteria are beneficial to the host (e.g., *Lactobacillus* and *Bifidobacterium*) and likely aid the adaptation of the host for prolonged periods (Matsumiya et al. 2002; Milani et al. 2015).

In some cases, such coevolution signal is so strong that the evolution path of a bacterium mirrors that of its host (Linz et al. 2007; Brooks et al. 2016). Such phylosymbiosis provides an alternative to reconstruct the animal evolutionary history, and ancient microbes can sometimes serve as a timestamp for temporal calibration of molecular rates of evolution. For example, the history of human migration recovered from microbes that colonize the human body and human mitochondrial genomes is strikingly concordant (Linz et al. 2007; Comas et al. 2013). Although modern *Helicobacter pylori* population supports the out-of-Africa theory, a 5,300-year-old *H. pylori* genome pinpoints the timing of the arrival of African population in Europe (Linz et al. 2007; Maixner et al. 2016). Nevertheless, some microbes preserve stronger coevolutionary signal than others. In hominid gut microbiota, the phylogeny of Bacteroidaceae and Bifidobacteriaceae parallels their hosts', whereas those of other gut species do not (Groussin et al. 2017).

Methods and Progress in Ancient Microbiome Research

Although the evolutionary history of a microbiome can be reconstructed by examining similarities and differences in the microbiome from related species (Moeller et al. 2014), a temporal record is essential to draw an accurate picture of how microbiota contribute to animal adaptation to the environment. Recent advances in paleomicrobiology provide powerful tools for examining the coevolutionary history of animals and their microbiota from alternative sources (Darling and Donoghue 2014; Warinner, Speller, and Collins 2015b). Currently, paleomicrobiome information comes from two main sources: fossilized feces (coprolites) and dental calculus, which reflect gut and oral microbiomes, respectively (Warinner, Speller, Collins, and Lewis 2015; Warinner, Speller, and Collins 2015b).

Animal dung that is quickly desiccated or covered by clay sediment can be preserved as a coprolite (McAllister 1985). Coprolites contain food remnants (such as plant debris, pollen, or prey skeletal elements), host DNA, gut microbial DNA, and DNA originated from the environment (Tito et al. 2012; Wood and Wilmshurst 2013; Rawlence et al. 2016). Several studies have recovered bacterial, fungal, and archaeal information from human and animal coprolites (van Geel et al. 2011; Wood et al. 2012; Santiago-Rodriguez et al. 2013; Boast et al. 2018), alongside digested and undigested food used to infer the diet of the host. However, this method is not without limitations. Because feces are rich in organic material that can be used by microbes after deposition, the microbial community continues to change after defecation. For example, the microbial community within a coprolite from a Latin American mummy resembled that from a modern compost pile, rather than that of the human gut (Tito et al. 2012). Additionally, coprolites are susceptible to contamination from the surrounding environment. Thus, the ancient gut microbiome information obtained from coprolites is usually biased and heavily subject to environmental contamination (Warinner, Speller, Collins, and Lewis 2015). Furthermore, feces typically decay rapidly and very few of them can be fossilized and preserved over a long time period, which makes the coprolite record more broken and incomplete than skeletal records (Warinner, Speller, Collins, and Lewis 2015). Although host genetic information can be obtained from coprolite in some cases (some gut epithelial cells might be present in the feces and provide host DNA), the lack of skeletal evidence of a specific host makes it difficult to identify a coprolite's origins.

In contrast to coprolites, dental calculus (calcified matrix formed from a biofilm on the teeth surface) is frequently found on the surface of ancient human teeth and is a more accessible source material than coprolites for recovering the evolutionary history of microbiomes (Lieverse 1999; Jin and Yip 2002; Warinner, Speller, and Collins 2015b). Calcified and noncalcified bacteria have been observed in dental calculus using transmission electron microscopy and gold-labeled antibodies (Warinner, Speller, and Collins 2015b). In addition, dental calculus provides an environment suitable for the preservation of ancient microbial DNA (Warinner, Speller, and Collins 2015b). Several studies have shown that microbial DNA can be successfully extracted and amplified from human dental calculus (Adler et al. 2013; Warinner, Speller, and Collins 2015b; Weyrich et al. 2017). Adler et al. (2013)

introduced high-throughput sequencing into a paleomicrobiome study and collected the first detailed genetic data from oral microbiomes of 34 ancient European human remains (Adler et al. 2013). They obtained ancient oral microbiome profiles using 16S ribosomal RNA gene amplicon sequencing techniques and observed a shift in the microbiome linked to dietary alterations. Several studies have since identified biases in using 16S ribosomal RNA gene amplicon sequencing, which is heavily subject to taphonomic and amplification biases (Ziesemer et al. 2015; Weyrich et al. 2017). Shotgun sequencing is now accepted as the gold standard to reconstruct ancient microbiomes (Ziesemer et al. 2015). Shotgun libraries include a subsample of all the DNA fragments instead of just a prokaryotic genetic marker. Shotgun libraries can capture eukaryotic DNA, including DNA from dietary food sources, pathogens, and the host. For example, DNA was obtained from potential food items, such as pork and wheat (Warinner et al. 2014) or rhinoceros and mushrooms (Weyrich et al. 2017). Oral microbiota reconstructed from a shotgun library can also be indicative of ancient lifestyles and diets. Variation of the Neanderthal microbiota was suggested to be associated with meat consumption (Weyrich et al. 2017), whereas microbiota consistent with a diet high in carbohydrates was observed in Neolithic and Medieval individuals (Adler et al. 2013). Shotgun data can also be used to reconstruct the microbial functions present within the microbiome and assemble the draft genome of commensal microorganisms. The abundant information can be used to infer ancient animal diet, behavior, and disease, as well as the interaction between ancient microbiota and their host (Baker et al. 2017; Weyrich et al. 2017). Nevertheless, amplicon sequencing is more cost-effective than shotgun sequencing and can still provide important microbiome information in terms of the presence or absence of taxa—but not relative or absolute abundance—due to the differential impact of degradation processes on microbial species (Boast et al. 2018).

These aDNA studies demonstrate the ability to accurately reconstruct animal microbiome records across evolutionary timescales, and thereby to investigate the interactions of microbiome, animal, and environment, revealing the roles that the microbiome may play in animal adaptation. Notably, dental calculus deposits are rare on most nonhuman mammals, but it is very likely that some oral microbiome information can be obtained from ancient mammal tooth specimens (e.g., similar, noncalcified biofilms formed by oral microbiome or food debris preserved in the occlusal surfaces and gaps of mammalian herbivore teeth). In addition to ancient microbial community, specific pathogens (e.g., *Yersinia pestis* and *Mycobacterium tuberculosis*) identified from ancient samples also have the potential to reveal epidemic events in the past that had immense impacts on ancient animals (Scott 1988; Bos et al. 2011). As current research is mainly limited to ancient humans, we advocate here that an emphasis should be placed on ancient microbiome research in other animals as well.

Dynamics among Epigenome, Microbiome, and Environment

The external factors that shape epigenetic modifications and the microbiome largely overlap (e.g., diet composition, lifestyle, and exposure to stressors). When animals are exposed to changing environments, it is highly likely that epigenetics and the microbiome respond to the stimuli simultaneously. Some phenotypic consequences of the alteration of epigenetics and the microbiome, such as embryonic development and immunity establishment, are crucial to the ability to survive an adverse environment. Unlike genetic adaptation, which is a long-term process, epigenetics and microbiome can respond rapidly to environmental cues (Yona et al. 2015; Alberdi et al. 2016). In particular, modifications that occur during the prenatal period or early stages of life can have life-long or even a trans-generational influence on animals (Cooney et al. 2002; Li et al. 2005; Anderson et al. 2012; Mueller et al. 2015).

Furthermore, emerging evidence suggests that the microbiome can directly interact with the host epigenome (Hullar and Fu 2014; Paul et al. 2015). Some pathogenic bacteria (e.g., *Mycobacterium leprae* or *H. pylori*) can induce epigenome modifications of the infected host cells, and sometimes even trigger epigenome-wide alterations (De Monerri and Kim 2014; Cizmeci et al. 2016). Global disruption of methylation reprograming has been detected in germ-free conditions (i.e., in the absence of a microbiome) (Yu et al. 2015), and the gut microbiome composition shows a clear association to host epigenomic profiles (Kumar et al. 2014). The crosstalk between epigenome and microbiome is not unexpected, as epigenetics plays an important part in shaping immunity (Amarasekera et al. 2013), which directly affects the community composition and ecology of indigenous microorganisms (Kau et al. 2011); conversely, the microbiome can release molecules (such as folate and transposases) that are directly or indirectly involved in the modification of the host epigenome (LeBlanc et al. 2013; De Monerri and Kim 2014). In this context, it is likely that epigenome, microbiome, and the environment form a complex and dynamic three-way interaction that could be the basis for rapid adaptation of animals to changing environments.

Epigenome and Microbiome Interactions with the Genome

Fast response of animal epigenomes and microbiomes to changing environments is the key to rapid adaptation discussed in this review. However, the observed changes in epigenomes and microbiomes do not necessarily originate independently of the host genome. It is also possible that genetic mutations cause subsequent alterations in epigenetics and microbiome (Jones 2012; Fulde et al. 2018). Such a case should not be considered as adaptation via epigenetic modification or microbiome alteration, because it fundamentally stems from the genome and will be much slower than the within-generation alterations of microbiome and epigenetics. Thus, in paleomicrobiome and paleoepigenetics studies, it is critical to be aware of three possible scenarios. First,

environmental changes directly triggered animal microbiome and/or epigenome alterations, in which no genetic alterations associated with these environmental changes should be detected. Second, genetic adaptation to the environment led to subsequent epigenetic and microbiome modifications. In this case, genetic changes with a causal link to the epigenetic and/or microbiome signatures should be detected. Third, both nongenetic and genetic adaptation occurred in response to environmental changes. The genetic and nongenetic adaptation can happen in parallel or sequentially (Yona et al. 2015). The difference between scenario 2 and scenario 3 is whether the alterations in epigenome and/or microbiome are triggered by genetic changes or environmental cues. This can be difficult to infer from ancient data directly. However, it is possible to use gene-editing techniques in animal models to test if the observed genetic variation can lead to the corresponding epigenetic and microbiomic changes.

The complex interactions between environment, genome, microbiome, and epigenome make animal adaptation to the environment an extremely complicated yet fascinating process. In order to recover a comprehensive evolutionary history, it is important to obtain epigenetic and microbiome information along with genomic information, as each factor will likely play a role in past animal adaptation.

Concluding Remarks

The role of epigenetics and the microbiome in animal adaptation has attracted increased attention in recent years, including the resurgence of two key theories: neo-Lamarckism and the hologenome theory of evolution (Zilber-Rosenberg and Rosenberg 2008; Skinner 2015; Danchin et al. 2019). It also raised extensive debate (Laland et al. 2014; van Opstal and Bordenstein 2015; Horsthemke 2018), which is mainly due to the limited understanding of both fields and inconsistency of experimental results in different animal models. There are several major challenges in current studies, including 1) the lack of a universally accepted animal model and experimental system that can serve as a gold standard, 2) the plasticity and tissue- and niche-specificity of epigenome and microbiome responses that make results very difficult to reproduce independently, and 3) experimental studies using modern animals can hardly be done over microevolutionary timescales (Rosenfeld 2010; Tripathi et al. 2018). Within this context, it is necessary to develop a model system in which both external- and internal-confounding factors are controlled and monitored, especially for the sake of elucidating the basic molecular mechanisms and dynamics underlying epigenetics and microbiomes.

On the other hand, numerous animal species that have experienced past climate and environmental turnovers provide an extraordinary repertoire for case studies. Although natural environments are more complex and less controlled than laboratory conditions, the availability of paleogenomic and paleoenvironmental data is fast accumulating, and recently developed approaches offer access to high quality paleoepigenome and paleomicrobiome data. Teasing apart the epigenomic, microbiomic, and genomic components in animal adaptation to environment might become increasingly feasible. More importantly, various animal species that have lived in diverse environments and have distinct evolutionary paths offer invaluable resources for future research.

Here, we propose that increasing efforts should be placed in paleoepigenomic and paleomicrobiomic research across the tree of life, including but not limited to 1) experimental and bioinformatics approaches further tailored for recovering epigenomic and microbiomic data from short and damaged DNA molecules, 2) applying cutting-edge approaches to retrieve paleoepigenomic and paleomicrobiomic data from nonhuman species, especially those for which a large number of subfossil specimens are available, 3) the generated data should be deposited into public repository with detailed metadata, and 4) pipelines should be developed to make data generated using different approaches comparable, such as paleoepigenomic data generated using aDNA damage profile and bisulfite sequencing, as well as paleomicrobiomic data generated using shotgun sequencing and amplicon sequencing. In conclusion, we believe that the combination of advanced aDNA techniques and increasing understanding of epigenome and microbiome will provide novel insights into animal adaption to rapidly changing environments in the near future.

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