DNA METHYLATION AND PHENOTYPIC VARIATION IN THREE NORTH AMERICAN LARGE MAMMALS

A Thesis Submitted to the Committee on Graduate Studies

In Partial Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Arts and Science

Trent University

Peterborough, Ontario, Canada

Environmental and Life Sciences M.Sc. Graduate Program

January 2025

ABSTRACT

DNA methylation and phenotypic variation in three North American large mammals

Natalie Czajka

DNA methylation (DNAm) is a useful indicator of phenotypic expression and diversity, and can potentially inform adaptations to environmental changes. This thesis uses epigenetic techniques to investigate the mechanisms underlying phenotypic variation in white-tailed deer, black bear, and mountain goat, with a particular focus on age and body size. In the second chapter, we aimed to contribute to wildlife monitoring by developing epigenetic clocks, or predictive models of age, and diagnostic markers of age class and sex. In the third chapter, we aimed to investigate the involvement of DNAm in body size variation of white-tailed deer by developing a model predictive of hindfoot length, and by identifying CpGs and genes that may be involved in hindfoot length variation. My results indicate that DNAm is an effective predictive marker of various phenotypes in these North American large mammals, and that epigenetic methods offer valuable insights for managing human impacts on wildlife.

Keywords: Epigenetics, DNA Methylation, Phenotypes, Age, Body Size, Population Genetics

ACKNOWLEDGEMENTS

I respectfully acknowledge that Trent University stands on the treaty and traditional territory of the Michi Saagiig Anishinaabeg. As a visitor on this land, I am grateful for the grounds on which I am able to conduct research and on which we are privileged to gather in the pursuit of higher education.

I first and foremost would like to highlight and thank my supervisors, Dr's. Aaron Shafer and Joseph Northrup, without whom this project never would have existed. I'm grateful for your continuous mentorship and support, the numerous R troubleshooting sessions, and for providing me the opportunity to do research that I value and deeply enjoy. This thesis encouraged me to venture into an unfamiliar field and led to invaluable personal and professional growth; thank you for believing in me as I navigated this project, and for being a constant source of inspiration. I would also like to extend my sincere thanks to my committee member, Dr. Catherine Cullingham, for her time, thoughtful comments, and valuable suggestions.

A special mention goes to my colleagues and friends in the Wildlife & Applied Genomics lab; I'm eternally grateful for their willingness to provide samples, help in the lab, share code, and go out for celebratory dinners. I'm particularly grateful to Brooklyn Cars and Marie-Laurence Cossette, who became my support system academically and beyond, especially when I was new to the city and felt like I had no idea what I was doing.

Most of all, I would like to thank my family; even though they didn't always understand my "deer stuff", their support throughout this degree was unwavering. Thank you for always encouraging me to do what I'm passionate about and being proud of me regardless of the outcome. I could not have completed this thesis without your love and support.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

length (x-axis, in units of centimetres) versus measured hindfoot length (y-axis, in unit of centimetres). The solid line indicates the linear regression with standard error indicated by the shaded area, and the dashed line depicts the diagonal (y = x). Cor represents the correlation coefficient (*r*), and MAE represents the median absolute error (in unit of years).

Figure 3. Manhattan plots of the Epigenome-wide association (EWAS) for hindfoot length in white-tailed deer. The coordinates are estimated based on the alignment of 34,070 Mammalian array probes to our white-tailed deer genome assembly. The direction of associations with p < 1.47e-6 (red dotted line) is highlighted by red (hypermethylated) and blue (hypomethylated) colours. Top 9 CpGs are indicated by their neighbouring genes.

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 1: GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

Human populations have been increasing globally, growing from 2 to 8 billion during the past hundred years, and the associated increase in human activity has directly and indirectly impacted wildlife and the environment (Cafaro et al., 2022; Crist et al., 2017). The modification of landscapes due to urbanization and other human-related disturbances drives habitat loss and fragmentation (Crist et al., 2017; Liu et al., 2016); natural habitats are converted for human use and often become unusable to wildlife, while major roads and railways reduce the connectivity of suitable habitats and isolate animal populations (Liu et al., 2016; Brearly et al., 2013). Humans also impact abiotic components of the ecosystem; greenhouse gas emissions, for example, are the result of human industrial activities and cause increasing air and water temperatures (He & Silliman, 2019). As human populations continue to grow and expand spatially, more direct impacts on wildlife are expected to occur as a result of increased interactions between humans and animals. These interactions include overhunting and increased disease transmission, both of which can decimate wildlife populations and impact biodiversity (Morrison et al., 2007; Brearly et al., 2013). There is therefore an increased need to mitigate these issues and to help conserve species biodiversity (McCance et al., 2017).

Human impacts on wildlife are widespread but not uniform; studies suggest that large mammals (>20 kg) are particularly susceptible to human disturbances (Morrison et al., 2007; Wan et al., 2019; Laliberte & Ripple, 2004). Large mammals require larger habitats and are therefore more impacted by habitat loss and fragmentation, and also tend to be preferentially hunted for nutrition and economic value (Morrison et al., 2007; Wan et al., 2019). Large

mammals also face an elevated risk of extinction, which is thought to be due to a variety of traits associated with increased vulnerability such as low reproductive rates (Cardillo et al., 2005). North American mammals specifically may be experiencing a decreased ability to cope with environmental challenges resulting from human activity, with harvested mammals experiencing a higher probability of maladaptation (Zheng et al., 2023). Large mammals are essential to ecosystem function and biodiversity as they regulate the availability of resources to other species within the ecosystem (Sinclair, 2003). This is accomplished by influencing plant and animal populations through predation and the modification of vegetation structure, consequently impacting the movement of nutrients and contributing to ecosystem health and function (Sinclair, 2003). Within North America, large herbivores such as bison and moose influence ecosystem composition through preferential grazing, regulating vegetation and creating habitat for other animals (Lacher et al., 2019; Leroux et al., 2020). On the other hand, carnivores such as wolves contribute through predation, culling prey species and providing food for species that feed on carcasses (Wilmers & Getz, 2005). Protecting large mammal species against the impacts of human activity is therefore essential for mitigating biodiversity loss and maintaining healthy ecosystems in the face of environmental change.

1.2 ENVIRONMENTAL ADAPTATION

Genomic data can be used to elucidate many population features, such as the presence of bottlenecks or inbreeding, population structure and size, and genetic variation (Willi et al., 2022; Hohenlohe et al., 2021). These parameters can be important for conservation efforts, as they inform what interventions may be necessary to preserve sustainable populations (Willi et al., 2022; Hohenlohe et al., 2021). Genomic data can also be used to investigate how wildlife is, or

has, adapted to environmental changes. However, genetic changes such as mutations typically accumulate across generations (Loewe & Hill, 2010); in large mammals, which tend to have long lifespans and generation times, environmental changes are occurring too quickly for adaptive responses that are reliant on the accumulation of mutations or other genetic changes (Hetem et al., 2014). Phenotypic traits on the other hand, are the observable traits (e.g., morphology and behaviour) exhibited by an individual and can be influenced by external factors such as environmental conditions and life history traits (Nachtomy et al., 2007; Tangili et al., 2023). Phenotypic diversity, or the variation in phenotypes within a population, allows for a response to changing environments. Some traits will confer increased fitness in new conditions, allowing subsequent generations to be better adapted to such conditions (McLean et al., 2012). For example, smaller body size is sometimes associated with increased heat tolerance; decreased growth within a population may therefore be a phenotypic change in response to increasing global temperatures (e.g., Brans et al., 2017; Elayadeth-Meethal et al., 2018). Ageing is another phenotype that has adaptive implications in a changing environment; a population with individuals who age and subsequently die off allows for the elimination of older individuals who may be less suited to a new or altered environment (Martins, 2011). Phenotypes can also be plastic, meaning a single genotype can produce a different phenotype in different environments (Kelly, 2019). Phenotypic plasticity itself can be an adaptive response, but it also plays a role in species adaptations to environmental changes through the alteration of which phenotypes are expressed in a population; this can include varying responses to cyclic or seasonal patterns, or the increased ability to acclimate to abiotic variables such as temperature or pH (Kelly, 2019). Phenotypes also act as indicators of several factors that are important to consider when developing and applying management and conservation interventions. For example, age and

ageing can inform processes such as survival, population growth, and harvest sustainability (Udevitz & Ballachey, 1998; Hecht, 2021; Harris & Metzgar, 1987; Milner et al., 2007).

As phenotypes are the observable manifestation of biological changes, understanding what mechanisms underlie phenotypic traits can provide insight into how large mammals may adapt to a changing environment and can inform the management and conservation of such species.

1.3 EPIGENETICS AND DNA METHYLATION

Epigenetic processes cause changes in gene expression that occur without altering a DNA sequence, and thus act as a bridge between genotype and phenotype (Bollati & Baccarelli, 2010). A common epigenetic modification is DNA methylation (DNAm). DNAm involves the transfer of a methyl group, typically to or from a cytosine (C) base within a DNA sequence through the actions of a family of enzymes called DNA methyltransferases (Jung & Pfeifer, 2015; Lyko, 2018; Moore et al., 2013). In mammalian genomes, this process often occurs at locations where a cytosine base is followed by a guanine (G) base, also referred to as cytosine-phosphate-guanine (CpG) sites (Moore et al., 2013). DNAm plays a role in gene expression; the addition of methyl groups often prevents the normal activation and expression of genes by blocking transcription, the process by which proteins are formed and genes are activated (Gibney & Nolan, 2010; Dhar et al., 2021; Gallego-Bartolomé, 2020; Jones et al., 2015). However, DNAm is a reversible modification, and the removal of a methyl group from a cytosine will once again make DNA accessible for transcription (Dhar et al., 2021; Unnikrishnan et al., 2019). Through its regulatory role in gene expression, DNA methylation influences what phenotype is expressed, underlying phenotypic diversity (Rosenfeld, 2010). Variations in DNAm patterns have already been linked

to traits such as ageing (Jin & Liu, 2018) and body size (e.g., Rzehak et al., 2017), two phenotypes that can inform environmental adaptations (Lenart & Bienertová-Vašků, 2017; Brans et al., 2017) and contribute information to wildlife management and conservation decisions.

DNA methylation undergoes a predictable decline across the genome with increased age (Moore et al., 2013; Dhar et al., 2021; Jones et al., 2015, Unnikrishnan et al., 2018). In mammals, this process is thought to result from a lack of DNAm preservation during cell divisions, the process by which tissues are grown and maintained; this lack of preservation results in a gradual loss of DNAm as individuals age and have increased occurrences of cell division (Jones et al., 2015; Teschendorff et al., 2013). Certain CpG sites within the genome also experience predictable site-specific increases or decreases in DNAm associated with increasing age (Jones et al., 2015; Unnikrishnan et al., 2019). Specific CpG sites where methylation reliably changes with age can be used as biomarkers, allowing for individual age prediction based on DNA methylation levels (Horvath & Raj, 2018; Unnikrishnan et al., 2019).

Variations in DNAm have also been linked to body size (e.g., Rzehak et al., 2017; Cao et al., 2015; Renard et al., 2022); however, body size is often cited as a polygenic trait, meaning it involves multiple genes, and the mechanism underlying this relationship likely varies between species (Posbergh & Huson, 2021; Makvandi-Nejad et al., 2012). Some studies contribute body size variation to differential methylation at genes involved in growth (e.g., Renard et al., 2022; Ma et al., 2019). Others also implicate global DNA methylation; for example, global hypomethylation in some species of ant larvae results in larger workers, while hypermethylation leads to smaller workers (Renard et al., 2022). In contrast, studies conducted in mammals found lower levels of DNA methylation in populations with smaller body sizes (Cao et al., 2015; Cossette et al., 2022).

1.4 STUDY SPECIES

White-tailed deer (*Odocoileus virginianus*), black bear (*Ursus americanus*), and mountain goat (*Oreamnos americanus*) are three species of North American large mammals, with population distributions ranging across Canada and the United States (Smith, 1991; Côté and Festa-Bianchet 2003; Scheick & McCown, 2014). These species hold cultural importance for many North American Indigenous peoples; all three species are harvested as part of traditional food sources (Parlee et al., 2021; Schuster et al., 2012; Tryland et al., 2018), and are also staples in traditional clothing, ceremonies, and symbolism (Samuel, 1990; Clark et al., 2021; Hewitt, 2015). These animals also contribute to local economies through multiple avenues. As big game species, these animals are sought after by recreational hunters and thus contribute to a multi-billion-dollar industry (Hewitt, 2015). These animals are also charismatic and are often sought out as a part of ecotourism (Honey et al., 2016; Hewitt, 2015; Vayro, 2023).

White-tailed deer, black bear, and mountain goat provide examples of phenotypic changes influenced by environmental variation, demonstrating the potential impact of human activity on large mammal species. White-tailed deer demonstrate strong phenotypic responses in body size directly related to population density and inversely related to food availability (Wolverton et al., 2009). These environmental factors are influenced by human activity through pathways such as hunting and habitat suitability (Marantz et al., 2016; Darlington et al., 2022). Female black bears living in proximity to urban areas are documented to have larger body sizes, likely due to the increased availability of alternative food sources (e.g., crops, garbage) (Nelson et al., 2024). Mountain goats are suggested to be particularly sensitive to environmental disturbances, and various studies document a relationship between availability and quality of vegetation and body size in kids (Côté & Festa-Bianchet, 2001; Pettorelli et al., 2007). Warmer

temperatures may therefore impact body size in this species through an influence on vegetation (Pettorelli et al., 2007). In addition to body size, climate also impacts variation in horn size (Martchenko et al., 2022). All three species also demonstrate age-related survival rates which in part result from environmental factors such as weather, predation, and hunting pressures (Delgiudice et al., 2006; Reynolds-Hogland et al., 2022; White et al., 2011). For example, older mountain goats seem to be more susceptible than younger individuals to harsh winter conditions (White et al., 2011), while white-tailed deer demonstrate a greater impact of predation on older individuals and a greater impact of hunting pressure on younger individuals (Delgiudice et al., 2006). These age-related effects emphasize the importance of understanding what underlies the interactions between age and environment, particularly when considering harvested and managed species.

The goal of this thesis is to investigate the epigenetic mechanisms underlying phenotypic variation in white-tailed deer, black bear, and mountain goat, with a specific focus on age and body size. In the second chapter, we quantified differential DNA methylation patterns at over 30,000 cytosine-guanine sites (CpGs) from tissue samples of all three species to develop epigenetic clocks, or predictive models of age. We also characterized individual CpG sites within each species that demonstrated clear differences in methylation levels between age classes and sex, which can be used to develop a suite of accessible diagnostic markers. We predicted that we would be able to identify CpG sites that are correlated to and predictive of age, and the aim of this chapter was to develop a tool with the potential to contribute to wildlife monitoring by providing easily obtainable representations of age structure in managed populations. In the third chapter of this thesis, we investigated the relationship between DNA methylation and hindfoot length in white-tailed deer. Here, we aimed to develop a model that is predictive of hindfoot

length, identifying CpGs predictive of this measure, and to identify genes that may be involved in hindfoot length variation. This chapter aimed to investigate the involvement of DNA methylation in body size variation and introduce new avenues for applying this relationship to wildlife management. Ultimately, the overarching goal of this thesis was to explore phenotypic traits from the epigenetic perspective, and to develop epigenetic tools that can aid and inform management decisions regarding three iconic North American large mammals.

CHAPTER 2: EPIGENETIC CLOCKS, SEX MARKERS, AND AGE-CLASS DIAGNOSTICS IN THREE HARVESTED LARGE MAMMALS

2.1 ABSTRACT

The development of epigenetic clocks, or the DNA methylation-based inference of age, is an emerging tool for ageing in free ranging populations. In this study, we developed epigenetic clocks for three species of large mammals that are the focus of extensive management throughout their range in North America: white-tailed deer, black bear and mountain goat. We quantified differential DNA methylation patterns at over 30,000 cytosine-guanine sites (CpGs) from tissue samples of all three species (black bear n = 49; white-tailed deer n = 47; mountain goat n = 45). We used a penalized regression model (elastic net) to build explanatory (black bear r = .95; white-tailed deer r = .99; mountain goat r = .97) and robust (black bear Median Absolute Error or MAE = 1.33; white-tailed deer MAE = 0.29; mountain goat MAE = 0.61) models of age or clocks. We also characterized individual CpG sites within each species that demonstrated clear differences in methylation levels between age classes and sex, which can be used to develop a suite of accessible diagnostic markers. This tool has the potential to contribute to wildlife monitoring by providing easily obtainable representations of age structure in managed populations.

2.2 INTRODUCTION

DNA methylation (DNAm) is an epigenetic modification primarily associated with the regulation of gene expression (Gallego-Bartolomé, 2020; Jung & Pfeifer, 2015). The process involves the transfer of a methyl group to or from a cytosine base (Jung & Pfeifer, 2015; Lyko, 2018; Moore et al., 2013). In mammalian genomes, this occurs primarily at cytosines that precede a guanine, also referred to as CpG sites (Moore et al., 2013). The role of DNAm in gene expression occurs at the transcriptional level where the addition of a methyl group is associated with chromatin condensation and the prevention of binding by transcriptional machinery, preventing the regular formation and activation of genes (Dhar et al., 2021; Gallego-Bartolomé, 2020; Jones et al., 2015). Likewise, the removal of a methyl group from a cytosine makes DNA available for transcriptional machinery and gene activation (Dhar et al., 2021; Unnikrishnan et al., 2019).

DNA methylation patterns have predictable changes with age (Moore et al., 2013); increasing age is associated with a global decline in DNAm (Dhar et al., 2021; Jones et al., 2015; Unnikrishnan et al., 2018). This process is thought to result from deregulation of DNAm during cell division and leads to an overall loss of DNAm as individuals age, or alternatively with the increased number of cell divisions (Jones et al., 2015; Teschendorff et al., 2013). Increasing chronological age can also be associated with site-specific increases or decreases in DNAm at predictable CpG sites within the genome (Jones et al., 2015; Unnikrishnan et al., 2019). Specific CpG sites where methylation reliably changes with age can therefore be used as biomarkers for chronological age, allowing for individual age prediction based on DNA methylation levels (Horvath & Raj, 2018; Unnikrishnan et al., 2019). The methylation-based inference of age, referred to as epigenetic clocks, is a conserved molecular mechanism across mammals (Lu et al.,

2023), but species and population differences in the molecular ageing process can arise due to external factors, specifically environmental conditions and life history traits (Tangili et al., 2023). Tissue-specific DNAm patterns also have been identified, with different tissues potentially reflecting different epigenetic ages (Jones et al., 2015). Collectively, this leads to universal, species-specific, and even tissue-specific epigenetic clocks (Lu et al., 2023; Robeck et al., 2021).

Epigenetic clocks confer many benefits when compared to traditional ageing methods in mammals, such as tooth section analysis, which are typically labour-intensive, invasive, and hard to implement at a large scale (Chinnadurai et al., 2016; Gasaway et al., 1978; Veiberg et al., 2020). DNAm data can be obtained from a variety of sources of DNA, including hair or faecal collection (Hao et al., 2021; Liu et al., 2021). Identifying a small number of CpG sites that strongly correlate with chronological age can facilitate the development of diagnostic markers, lowering processing costs of age estimation without sacrificing accuracy (Han et al., 2018). This approach has relevance for harvested and managed populations, where age information is often directly used to better understand processes such as survival, population growth and harvest sustainability (Harris & Metzgar, 1987; Hecht, 2021; Udevitz & Ballachey, 1998).

No species-specific epigenetic clock has yet been developed for white-tailed deer (Odocoileus virginianus), black bear (Ursus americanus) or mountain goat (Oreamnos americanus). These three North American species hold significant cultural importance for many North American Indigenous peoples, as all three are often harvested as part of traditional food sources (Parlee et al., 2021; Schuster et al., 2011; Tryland et al., 2018). These species also contribute significantly to local economies through their involvement in hunting and associated activities. All three species are the subject of intensive management throughout their range

(Hristienko & McDonald, 2007; McShea, 2012; Smith, 1988), and these efforts often rely on age information. Current ageing methods for these species offer various challenges, though all three can typically be aged to at least age class by diagnostic phenotypes such as antlers and body size. Mountain goats are most often aged by the number of horn annuli, which is challenging and less accurate in older animals (Stevens & Houston, 1989). Deer are typically aged by tooth wear, a method highly influenced by environmental conditions such as soil and vegetation type (Foley et al., 2021). Tooth section analysis is the preferred method for ageing bears (Harshyne et al., 1998), which is labour-intensive but accurate. Further, most current ageing methods require physical handling of the animal, while non-invasive ageing methods, such as using body size or antler characteristics during aerial survey, are coarse and can lead to biases due to reduced detectability of certain age classes or misidentification (Davis et al., 2022). In this study, we developed epigenetic clocks for white-tailed deer, black bear, and mountain goat by quantifying differential DNA methylation patterns across known chronological ages. We also characterized individual cytosine-guanine sites (CpGs) within each species that were highly correlated with age class and sex to develop a suite of accessible diagnostic markers.

2.3 METHODS

2.3.1 Sample collection and DNA extraction

We collected tissue samples from three species of North American large mammals, American black bear (n = 49), mountain goat (n = 45) and white-tailed deer (n = 47) sampled across Canada and the United States; collected tissues primarily consisted of skin (black bear) or a combination of skin and muscle (mountain goat and deer; Table 1; Data S1). Mountain goat and a subset of deer samples were collected by local managers, while some deer samples in

Ontario were provided voluntarily by hunters; these samples were stored immediately in ethanol. Bear tooth samples were provided by hunters via mail. All samples were then frozen upon arrival at the laboratory at −20°C until processing. Animals were aged to class in the field by hunters or managers, and chronological age was later estimated using either tooth section, tooth wear, horn annuli, or a combination (Data S1).

DNA was extracted from samples using the QIAGEN DNeasy Blood & Tissue Kit, following the manufacturer's standard protocol (Qiagen, Valencia, CA), and the concentration was measured using a QUBIT 3 fluorometer (Thermo Fisher Scientific). Samples with an extracted DNA concentration under 10 ng/μL were re-extracted or concentrated using a vacufuge. DNA was then standardized to 300 ng per 20 μL and plated in 96-well plates following an order determined using the R package Omixer version 1.6.0, which randomized plating order by covariates (i.e., age and sex) to minimize batch effects (Sinke et al., 2021). DNA was subjected to bisulfite conversion with the EZ-96 DNA methylation kit (Zymo Research, Irvine CA) and analysed using a large-scale Illumina methylation array (HorvathMammalMethylChip40) following the manufacturer's standard protocol (Illumina, San Diego, CA). Arrays were run on the iScan system, both in house (Jones Lab, University of Manitoba) and through a paid service (University of California, Los Angeles). The array quantifies DNA methylation levels at 37,492 CpG sites (Arneson et al., 2022) by measuring the methylated and unmethylated states of each CpG using a single probe with two colour channels; the methylated signal is measured by the intensity of a green colour channel, while the unmethylated signal is measured by the intensity of a red colour channel.

2.3.2 DNA methylation data and selection of species-specific CpGs

Raw DNA methylation data were provided as the intensity values for each CpG. These data were normalized and translated to beta values, defined as the ratio between methylated and unmethylated intensity, using the minfi normalization method (Fortin et al., 2017), version 1.42.0. The ComBat function from the R package sva version 3.44.0 (Leek et al., 2012), which applies parametric empirical Bayesian to account for batch effect, was applied (with age as an adjustment variable). For each species, the array probes were filtered to exclude CpGs that were not detected in the corresponding reference genome (black bear assembly accession no.—GCA_003344425.1; white-tailed deer—GCA_014726795.1; mountain goat—GCA_009758055.1). Here, species-specific CpGs were determined by aligning probes to each genome using QuasR version 1.12.0 (Gaidatzis et al., 2015); probe sequences that did not align were discarded from subsequent analyses.

2.3.3 Clock development and diagnostic CpGs

Epigenetic clock development followed the approach of Wilkinson et al. (2021). We created penalized regression models using elastic net regression within the glmnet R package version 4.1-6 (Friedman et al., 2010). A 10-fold internal cross-validation on the training set (black bear: n = 47, white-tailed deer: n = 33, mountain goat: n = 40) was used to determine the optimal penalty parameter (λ). In addition to fitting models to untransformed chronological age data, two different transformations were applied and models were compared to determine optimal linear fit using median absolute error: log-transformed (log[x + 1]) chronological age, and square-root-transformed (sqrt[x + 1]) chronological age. We performed a leave-one-out (LOO) cross-validation to obtain unbiased estimates of accuracy in regard to the DNAm age estimations, and we reported as estimates the correlation (r) between the DNAm age estimate

and estimated chronological age, and median absolute error (MAE), defined as the median absolute difference between predicted DNAm age and estimated chronological age. We also predicted DNAm age using the universal pan-mammalian epigenetic clock (Lu et al., 2023); three universal clocks were applied to each species based on a subset of probes, and the difference between predicted DNAm ages and chronological age was calculated (Δ). We compared Δ values from species-specific clocks to those from the universal clock.

Lastly, the calculated optimal penalty parameter was used to generate a list of specific CpGs that strongly correlated with age class in each study species. For each of these, DNA methylation levels across samples were plotted by age class: (i) white-tailed deer: fawn (0.5 years old), subadult (1.5–2.5 years), adult (>2.5 years); (ii) mountain goat: yearling (1 year), subadult (2–3 years), adult (>3 years); (iii) black bear: cub (1 year), subadult (2–4 years), adult (>4 years). We compared mean methylation levels between pairs of classes using a Wilcoxon test, and between all three classes using a Kruskal–Wallis test. Cytosine-guanine sites that showed significant differences in mean DNA methylation levels between age classes were selected as diagnostic CpGs ($\alpha = .05$). To identify CpGs diagnostic of sex, epigenome-wide association studies (EWAS) were conducted using the limma package v.3.56.2 (Ritchie et al., 2015); here, we used the normalized beta values of aligned probes with age and sex as fixed effects. The most significant CpG identified by the model was extracted from the data set, and we compared mean methylation levels between sexes using a Wilcoxon test.

2.4 RESULTS

2.4.1 Species-specific epigenetic clocks and diagnostic CpGs

A total of 21 samples across species were excluded from clock development due to quality control metrics (Data S1). The independently constructed clocks included samples from the remaining 120 individuals: 47 black bear samples, 33 white-tailed deer and 40 mountain goat samples. The chronological age of samples ranged 0.5–10.5 years in white-tailed deer, 1–12 years in mountain goats and 1–19 years in black bears. Of the 37,492 probes used in the methylation array, 33,751 probes were aligned to the black bear genome, 34,070 to white-tailed deer and 31,655 to mountain goat.

The clock using the log-transformed age model yielded the highest accuracy across species, demonstrating the lowest median absolute error (Figure S1). Based on λ, each age prediction model identified a subset of CpGs that were predictive of age in the different species: 38 CpGs in white-tailed deer (λ = .116), 38 in mountain goat (λ = .143) and 30 CpGs in black bear (λ = .297; a list of all significant CpGs and associated genes can be found in Data S2). These final log-transformed species-specific clocks were predictive of chronological age: (i) black bear: r = .95, median absolute error or MAE = 1.33 years; (ii) white-tailed deer: r = .99, MAE = 0.29 years; and (iii) mountain goat: r = .97, MAE = 0.61 years (Figure 1).

DNA methylation levels of the CpGs used in the species-specific clock were grouped by age class and Kruskal–Wallis and Wilcoxon statistical tests were performed (Figures S2–S4); clear differences in mean DNAm level were found between all pairs of age classes in eight black bear CpGs, four mountain goat CpGs and two white-tailed deer CpGs. The three CpGs in each species demonstrating the largest differences between age classes are shown (Figure 2a,c,e). Methylation levels according to sex were plotted for the most significant CpG identified by our EWAS model for each species, and a pairwise comparison between sexes was conducted using a Wilcoxon statistical test; significant differences were found between the mean methylation levels

of each sex for all three species (Figure 2b,d,f; a list of the most significant CpGs per species and associated genes can be found in Data S3).

2.4.2 Universal clock comparison

We applied three previously published universal pan-mammalian clocks to each species and compared the resulting predicted age to known chronological age to determine accuracy (Lu et al., 2023). Of these three clocks, Clock 1 performed poorly, reflected in high Δ values and negative age predictions (Data S4). Mean Δ values were lowest for Clock 2 and were as follows: 2.27 for black bear, −0.83 for white-tailed deer, and −2.66 for mountain goat (Figure 3a–c). Species-specific clocks showed lower Δ values (Figure 3d–f).

2.5 DISCUSSION

We report the development of novel species-specific clocks for three harvested large mammals in North America: white-tailed deer, black bear and mountain goat. We also identified individual diagnostic CpGs for age-class and sex that negate the need for a genome-wide array, which would greatly increase accessibility and decreases cost. These identified CpGs could be adapted to bead-based genotyping (Sato et al., 2011), pyrosequencing (Fleckhaus & Schneider, 2020) or qPCR assays (Bendixen et al., 2023), even bisulfite Sanger sequencing is an option (Brisotto et al., 2015). Further, the developed species-specific clocks represent a tool for estimating the age of these three species with low error and invasiveness, while circumventing reliance on diagnostic phenotypes. Importantly, these clocks can be applied to samples that cannot be aged using traditional methods (e.g., a mountain goat with broken horns or butchered and processed animals).

The species-specific clocks demonstrated reduced error in predicted ages when compared to the published pan-mammalian epigenetic clocks, which is consistent with the literature that suggests species-specific clocks improve the accuracy of age predictions (Peters et al., 2023). This finding is likely reflective of models built specific to the species—only a small number of CpGs overlapped with those used in the universal clocks (Data S2)—which excludes >300 CpGs used in universal clocks including those not present in a species genome. While the clocks we developed share similar patterns to other mammals (e.g., Caulton et al., 2021; Robeck et al., 2023), the residuals appear to increase with age, notably in bears and mountain goats, suggesting reduced accuracy in older individuals. This is possibly due to error in the chronological ages used in our model. Age-related decline in the accuracy of traditional ageing methods is common in all three study species (Foley et al., 2021; Harshyne et al., 1998; Stevens & Houston, 1989; Storm et al., 2014). External factors such as disease (e.g., Bobak et al., 2022), inbreeding (Larison et al., 2021) and stress (Pacht et al., 2021; Zannas et al., 2015) can also influence biological ageing in the form of DNAm and could also be contributing uncertainty to the model.

Tissue samples used for the development of our clocks were predominantly skin for black bear, with a mixture of skin and muscle for mountain goat and white-tailed deer. Epigenetic clocks calibrated to different tissue types can differ in accuracy and predicted DNAm age since methylation patterns differ among tissues (e.g., Robeck et al., 2021). The mixture for tissue types would produce a pan-tissue clock for mountain goat and deer (e.g., Lu et al., 2023), but tissue types would need to match those in black bear in order to properly apply our developed clocks. All tissue types collected in this study are typically collected by managers and field researchers, so this should not limit future application.

2.5.1 A tool for wildlife monitoring

Epigenetic clocks, once built, provide a reproducible and accurate tool for age prediction that has important potential for use in wildlife management. Identifying the CpGs predictive of age (<40 in our case) allows for the later development of assays that could be implemented at a relatively large scale. For example, many regions use barbed-wire hair snares for bear population estimates (Beier et al., 2005; Kendall et al., 2008); it is conceivable that with the same sample used for individual identification, a targeted DNAm assay could provide age and sex information on a population scale (e.g., Hao et al., 2021). Epigenetic clocks from hair samples, for example 10–20 follicles (Hao et al., 2021), have successfully been developed in animals such as cattle and demonstrate an accuracy not much lower than those developed using biopsy-based samples (Hayes et al., 2021). Assaying a small number of CpG sites can reduce costs and equipment requirements, creating the potential for more rapid and accessible ageing tools (e.g., Polanowski et al., 2014). Here, qPCR techniques are quite promising given their sensitivity and accessibility (Bendixen et al., 2023).

The use of age structure information is key in achieving one of the primary goals of wildlife management, which is to maintain harvested populations at sustainable levels. This is often accomplished by limiting or focussing harvest on specific age classes and sexes (Milner et al., 2007), which benefits from reliable data on population age structure that can be difficult or expensive to obtain. For example, the inclusion of calves and yearlings to the harvest quota of moose in Norway decreased pressure on adult females; the associated increase in average female age led to increased fecundity and population growth (Solberg et al., 1999). In other situations, the selective harvest of older individuals (i.e., those experiencing reproductive senescence) can increase the reproductive rate of a population (Milner et al., 2007). Epigenetic clocks could be

used to monitor, or augment monitoring, by providing accurate estimates of population age structure, which can then be used to determine whether such management initiatives are warranted and to monitor their success. These clocks also may offer new avenues for developing robust estimates of age structure for species that are difficult or impossible to monitor in other ways.

TABLES AND FIGURES

Table 1. Basic sample information, including species common and Latin names, age ranges of samples (in years), locations of sample collection, and number of individuals from each sex.

Common Name	Latin Name	Age Range	Females	Males	Tissue Type	Location
American black bear	*Ursus americanus*	1 - 19	24	25	Skin, Fibroblast	Ontario, Canada
White-tailed deer	*Odocoileus virginianus*	0.5 – 10.5	21	26	Skin, Muscle, Blood	Ontario, Canada; Texas, USA
Mountain Goat	*Oreamnos americanus*	1 – 12	22	23	Skin, Muscle, Larynx	Alaska, USA; Washington, USA

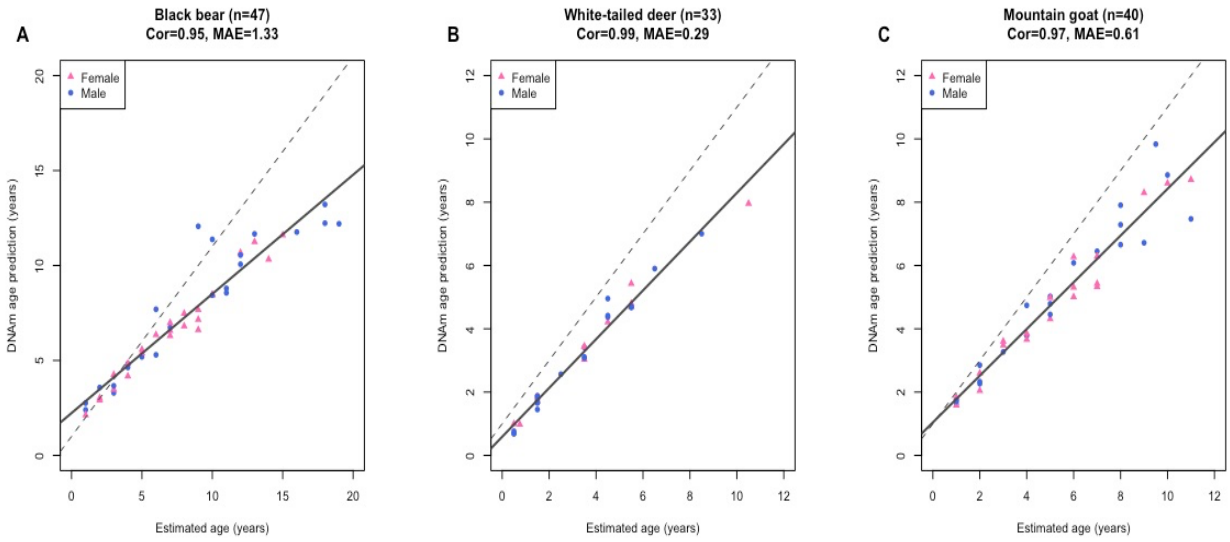


Figure 1. Leave-one-out cross-validation study of species-specific epigenetic clocks for **A** black bear, **B** white-tailed deer, and **C** mountain goat. DNAm age prediction (y-axis, in units of years) versus estimated chronological age (x-axis, in unit of years). The solid line indicates the linear regression of epigenetic age, and the dashed line depicts the diagonal (y = x). Cor represents the correlation coefficient (*r*), and MAE represents the median absolute error.

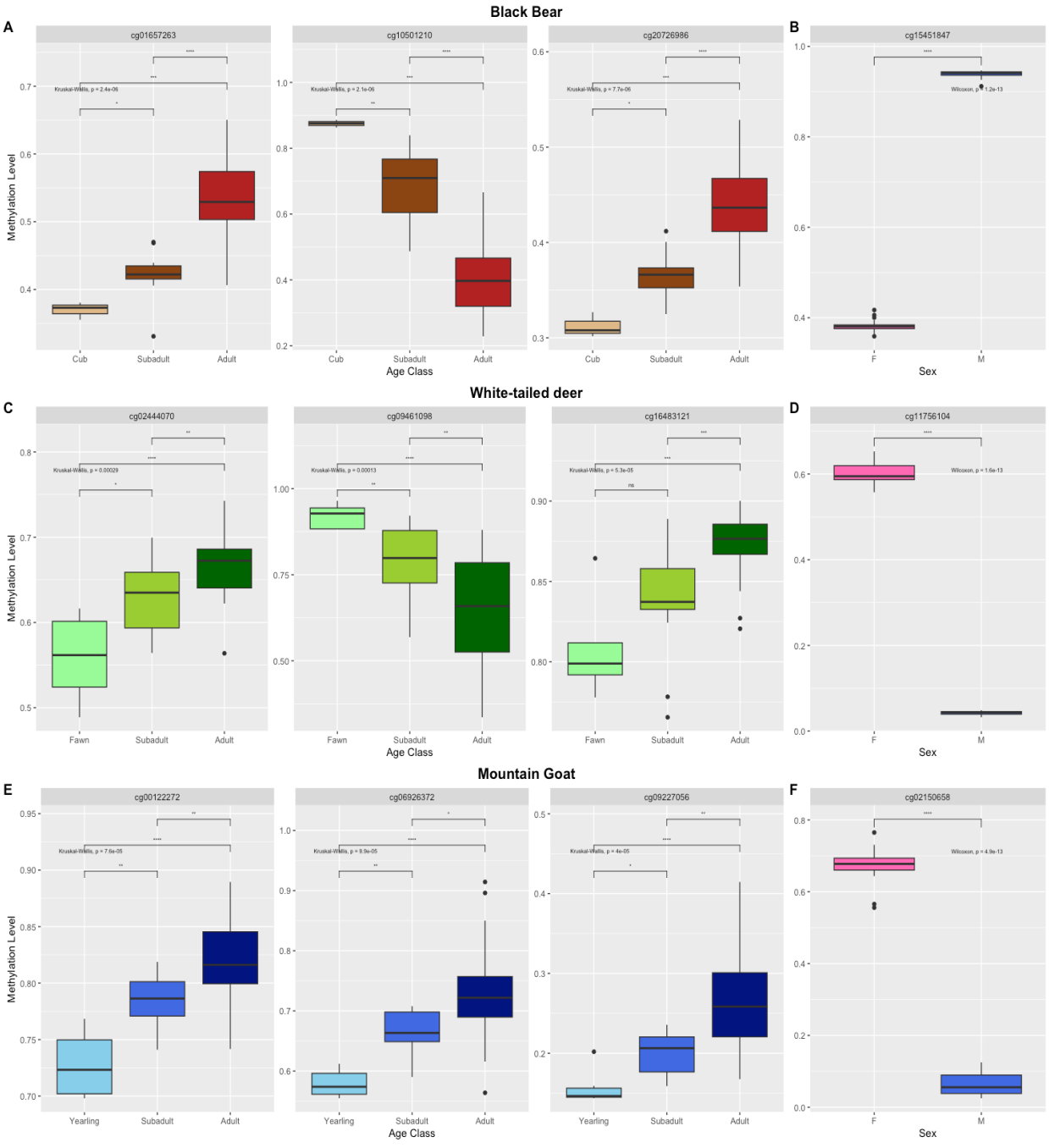


Figure 2. Boxplot of methylation level across age classes (**A, C, E**) and sex (**B, D, F**) at specific CpGs in black bear, white-tailed deer, and mountain goat. P-value significance level for pairwise comparisons (Wilcoxon test) is represented by asterisks (*). P-value for the comparison between all three age classes (Kruskal-Wallis test) and between sexes (Wilcoxon) is reported for each CpG.

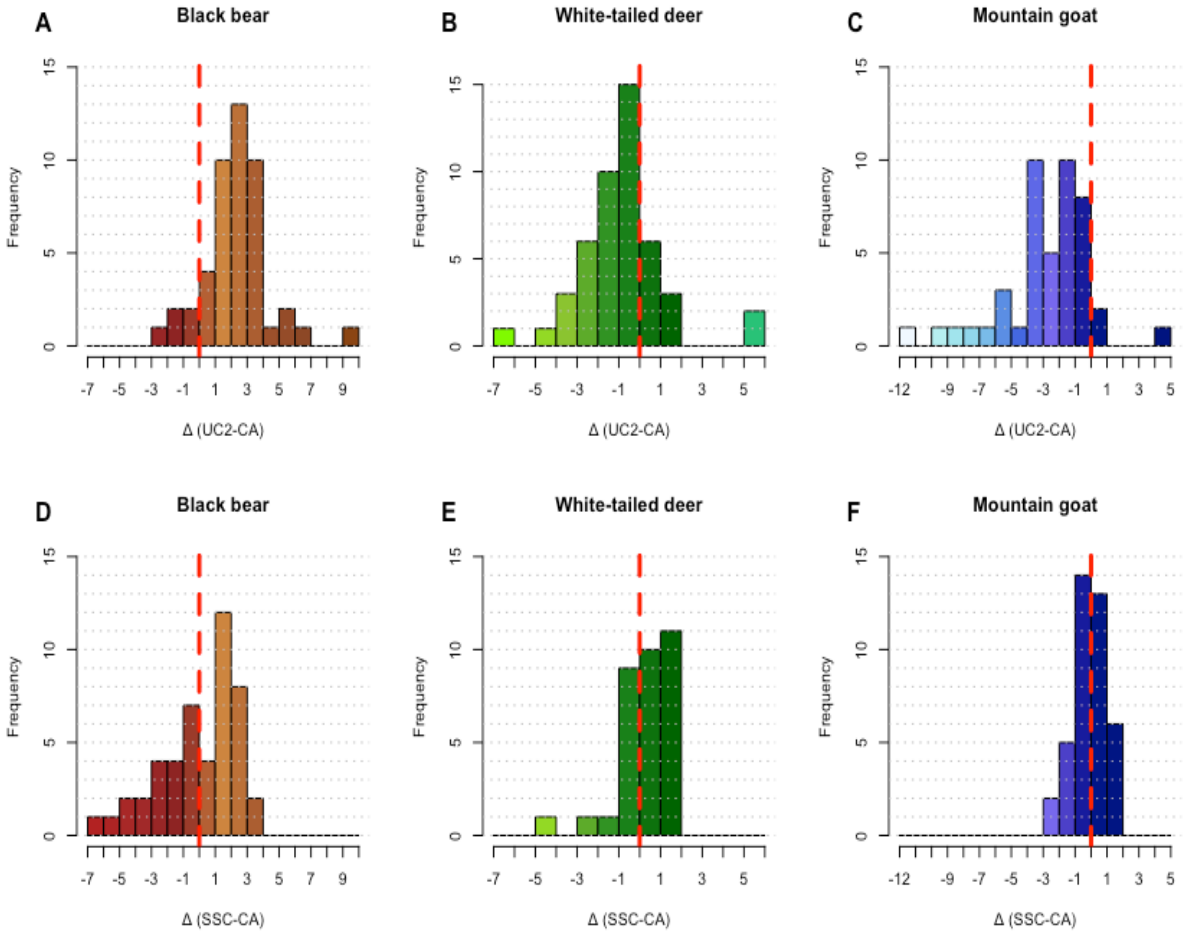


Figure 3. Comparison of Δ values (predicted DNAm age - chronological age) calculated from the universal pan-mammalian Clock 2 (**A – C)** and the species-specific clocks (**D – F**). The dashed red line represents a value of 0 (no difference between ages).

CHAPTER 3: INVESTIGATING BODY SIZE VARIATION IN WHITE-TAILED DEER AND THE INFLUENCE OF DNA METHYLATION

3.1 ABSTRACT

Body size is an important phenotypic measure for investigating responses to environmental or evolutionary pressures, as it serves as a simultaneous manifestation of short-term responses and long-term adaptations of organisms to their environments. Changing body sizes indicate a species' response to environmental changes such as climate warming, food web resilience, and habitat quality. The relationship between phenotypic variation and gene expression allows for this body size variation to be investigated through the analysis of DNA methylation. In this study, we quantified differential DNA methylation patterns at over 30,000 cytosine-guanine sites (CpGs) from tissue samples (n=148) taken from white-tailed deer of varying age classes and applied a previously established species-specific epigenetic clock to estimate age. We then used a penalized lasso regression to develop a model that is predictive of hindfoot length in white-tailed deer (r = 0.88, MAE = 1.31). We also identified 9 specific CpG sites that are associated with genes that may be involved in hindfoot length variation. This study supports the involvement of DNA methylation in body size variation and introduces new avenues for applying this relationship to wildlife management.

3.2 INTRODUCTION

Whereas genotype refers to static states of DNA molecules, phenotype typically encompasses observable traits (e.g., morphology and behaviour) that can be influenced by external factors such as environmental conditions and life history traits (Nachtomy et al., 2007; Tangili et al., 2023). The variation within a phenotype, or phenotypic diversity, plays an important evolutionary role; populations can respond to an environment by favouring traits that increase fitness at the individual level, thus enabling adaptations to novel and changing conditions (McLean et al., 2012). This leads to the prevalence of more tolerant phenotypes, or those that are better adapted to the new conditions. Understanding the mechanisms that drive and influence phenotypic variation is therefore important for understanding the evolution of individual traits and for predicting species' responses, and potential for responses, to environmental change. This is particularly relevant as human activity, and the associated impacts on wildlife and the environment, is increasing and leading to drastic ecological changes such as increasing air and water temperatures (Cafaro et al., 2022; Crist et al., 2017; He & Silliman, 2019).

While external factors such as environmental conditions influence phenotypic variation, drivers of this variation can also occur at the molecular level. Gene expression is a fundamental biological process that involves the transcription and translation of genetic information to produce essential cellular components such as proteins (Gibney & Nolan, 2010). The regulation of gene expression involves the interaction of numerous mechanisms across various biological processes (Gibney & Nolan, 2010). One such regulatory mechanism is DNA methylation (DNAm), an epigenetic process involving the transfer of a methyl group typically to or from a cytosine base (Jung & Pfeifer, 2015; Lyko, 2017; Moore et al., 2013). Within mammalian

genomes, this chemical modification predominantly occurs at sites within the genome where a cytosine base is followed by a guanine base, referred to as CpG sites (Moore et al., 2013). The interaction between DNAm and gene expression is largely at the transcriptional level, as the addition of methyl groups obstructs the normal activation of genes by impacting chromatin condensation and the binding of transcriptional machinery, thus leading to transcriptional silencing (Gibney & Nolan, 2010; Dhar et al., 2021; Gallego-Bartolomé, 2020; Jones et al., 2015). DNAm is also a reversible process; gene activation can be re-attained through the removal of a methyl group from a cytosine, once again making DNA accessible to transcriptional machinery (Dhar et al., 2021; Unnikrishnan et al., 2019). The role of DNAm in gene expression is taxonomically widespread and has been documented in groups such as mammals, fish, and amphibians (Yagi et al., 2008; Anastasiadi et al., 2018).

By regulating gene expression without altering the underlying DNA sequence, DNAm is also implicated in phenotypic diversity (Rosenfeld, 2010). The analysis of DNAm therefore not only contributes to our understanding of phenotypic diversity, but also offers insight into what processes drive evolution. Studies have already begun to investigate the relationship between DNAm and phenotypic traits, with large-scale studies investigating the influence of DNA methylation in mammalian traits (Haghani et al., 2023). Variations in DNAm patterns have been linked to traits such as disease and aging (Jin & Liu, 2018), body temperature (e.g. Varriale & Bernardi, 2006), and body size and composition (e.g. Rzehak et al., 2017; Cao et al., 2015; Ma et al., 2019).

Body size (length, mass etc.) is a phenotype that is strongly influenced by environmental variation; for example, smaller body size is sometimes associated with increased heat tolerance, potentially informing phenotypic change in response to increasing global temperatures (e.g.,

Brans et al., 2017; Elayadeth-Meethal et al., 2018). These patterns also apply across taxa, with larger mammalian species typically occupying colder climates and higher latitudes (Blackburn & Hawkins, 2004). One hypothesis for this phenomenon is that a large body size increases heat conservation due to a lower surface area to volume ratio (Blackburn & Hawkins, 2004). Body size can also indicate detrimental changes in populations and ecosystems; changing body size can negatively alter the resilience of the food web to disturbance, and suggests future population collapse (Woodward et al., 2005; Clements & Ozgul, 2016). Understanding variation in body size and the underlying mechanisms driving this variation is therefore important for predicting how a species may respond to rapid environmental change, such as that from climate warming. Large mammals are a good group within which to study these interactions, as they play a significant role in ecosystem function by regulating the availability of resources to other species, and are disproportionately impacted by ecological disturbances (Sinclair, 2003; Cardillo et al., 2005). North American mammals may be experiencing a decreased ability to cope with environmental challenges, with harvested mammals in particular experiencing a higher probability of maladaptation (Zheng et al., 2023).

White-tailed deer (*Odocoileus virginianus*) are large, free-ranging North American mammals that play a significant role in Indigenous cultures through their harvest as a traditional food source, while also contributing to local economies through their involvement in sport hunting and related activities (Parlee et al., 2021). White tailed deer also offer an interesting case study when investigating environmentally mediated body size variation, as they demonstrate strong phenotypic responses to population density and food availability (Wolverton et al., 2009). Lastly, white-tailed deer act as keystone herbivores and are the subject of intensive management throughout their range (Waller & Alverson, 1997; McShea, 2012); understanding body size

variation and associated drivers in this species can therefore potentially inform management decisions and interventions. In this study, we examined the contribution of DNAm to body size variability in white-tailed deer and investigated potential pathways underlying this relationship, where hindfoot length serves as the proxy for body size. This study utilizes the epigenetic perspective to offer novel insight on body size diversity in free-ranging mammal populations.

3.3 METHODS

3.3.1 Sample Collection & DNA Extraction

White-tailed deer (hereafter deer) were captured using modified Clover traps (Clover, 1954), which were monitored via remotely triggered cellular cameras (Spypoint Inc., model link-micro-lte). Ear tissue samples were taken using a 5-millimetre biopsy punch, and samples were frozen upon arrival at the lab at -20°C until processing. Deer were sexed and aged to class in the field, and hindfoot measurements were collected prior to release (Table 1). More information on field methods can be found in Sucharzewski (2024). The QIAGEN DNeasy Blood & Tissue Kit was used to extract DNA from ear tissue samples, following the manufacturer's standard protocol (Qiagen, Valencia, CA). The concentration of extracted DNA was determined using a QUBIT 3 fluorometer (Thermo Fisher Scientific); if the concentration was quantified as < 15 ng/µl, DNA was re-extracted or concentrated using a vacufuge. We prepared DNA for plating in 96-well plates by standardizing to 300 ng per 20 µl and randomizing plating order by covariates (i.e., age class, sex) using the R package Omixer version 1.6.0; this was done to minimize batch effects (Sinke et al., 2021).

3.3.2 DNA methylation data

Bisulfite conversion of the DNA was performed using the EZ-96 DNA methylation kit (Zymo Research, Irvine CA), and a large-scale Illumina methylation array (HorvathMammalMethylChip40) was run on the iScan system following the manufacturer's standard protocol (Illumina, San Diego, CA). This array uses a single probe with two colour channels per CpG site to quantify DNA methylation levels at a total of 37,492 sites (Arneson et al., 2022). We used the minfi normalization method (Fortin et al., 2017), version 1.42.0 to normalize and translate raw data to beta values, meaning the ratio between methylated and unmethylated intensity. The ComBat function from R package sva version 3.44.0 (Leek et al., 2012), which applies parametric empirical Bayesian to account for batch effect, was applied (with age class as an adjustment variable). Array probes were aligned to the white-tailed deer reference genome (GCA_014726795.1) using QuasR version 1.12.0 (Gaidatzis et al., 2015); methylation data associated with any probes that did not align was excluded from subsequent analyses.

3.3.3 Biological age prediction and genotypic PCA

Czajka et al. (2024) previously developed and published a white-tailed deer epigenetic clock using elastic net (Lu et al., 2023) to reduce the >30,000 CpG sites to 38 sites that were highly predictive of age class. To predict biological age in our current sample, methylation data for all samples was filtered to only those CpG sites identified by Czajka et al. (2024). We then used the elastic net model to estimate age from methylation data, and compared mean predicted age between pairs of age classes using a Wilcoxon test, and between all three classes using a Kruskal–Wallis test. Samples were genotyped at 16 loci (Cullingham et al. 2011), and a principal component analysis (PCA) via distance matrix was performed on genotype data to quantify the

distribution of genetic variation (McVean, 2009). The first two components of the PCA analysis were included as variables in our models.

3.3.4 Model creation and EWAS

We created penalised regression models using lasso regression within the glmnet R package version 4.1-6 (Friedman et al., 2010). These models aimed to identify variables predictive of hindfoot length and included: sex, age class, predicted biological age from the elastic net model of Czajka et al. (2024), capture month, all 34,070 deer-specific CpG sites, and the microsatellite principle components 1 and 2 as predictive variables (Table 2). All models were applied to samples for hindfoot length predictions; beta values for each CpG were multiplied by the variable coefficient determined by the model. The exponential of these multiplied values was then computed to convert methylation values to a hindfoot length measurement. We performed a leave-one-out (LOO) cross validation to obtain estimates of precision and accuracy for predictions of hindfoot length. We reported median absolute error (MAE), defined as the median absolute difference between predicted hindfoot length and measured hindfoot length, and Pearson correlation (r) between measured and predicted hindfoot length. The lowest MAE and highest r value would indicate the most highly predictive model.

To identify genes most highly associated with hindfoot length, an epigenome-wide association study (EWAS) was conducted using the limma package v.3.56.2 (Ritchie et al., 2015). We used the normalized beta values of aligned probes to run the following model: CpG methylation ~ hindfoot length (log) + sex + predicted biological age. A Bonferroni-corrected p-value threshold of < 1.47e-6 was set as the conservative cut-off for significance (Johnson et al., 2010), and the human Hg19 genome annotation was used to identify genes associated with the significant CpGs from the EWAS model.

3.4 RESULTS

3.4.1 Biological age prediction and PCA

A white-tailed deer-specific epigenetic clock comprised of 38 CpG sites (Supplemental Table 1) was applied to all collected samples (n = 148) with the output being predicted biological ages ranging from 2.28 – 7.99 years (Supplemental Data File S1). Kruskal-Wallis and Wilcoxon tests were performed; clear differences in mean predicted age were found between all pairs of age classes (Figure 1). PCA was applied to genotype data (Supplementary Data File S2); the first two components were included as variables in our models, where the first principal component (PC1) explained 6.66% of the total variation, and the second principal component (PC2) explained 5.97% of total variation.

3.4.2 Model outputs and EWAS

We constructed three models using data only from individuals with recorded hindfoot measurements (n = 123) (Table 2). Model 3 excluded age and sex as predictive variables; to account for potential effects of age and sex, we applied this model to subset sample groups: adult males, adult females, fawn males, and fawn females. Based on variables with non-zero coefficients, all models identified CpG sites predictive of hindfoot length (Supplemental Table 2). Model 3 demonstrated the lowest MAE and highest correlation when compared to known hindfoot length (Figure 2a; for all hindfoot length predictions see Supplemental Table 3). Although age and sex were not included in Model 3, some of the extracted CpGs are correlated with sex and age (Supplemental Figure 1-3). The age- and sex-subset models 3.1-3.4, meaning those that were applied to subset sample groups, showed hindfoot length tended to be over-estimated in adults and under-estimated in fawns (Figure 2b-e).

In the EWAS analysis, 9 of the 34,070 included CpG sites showed statistically significant (p-value < 1.47e-6) methylation; of these, 4 sites were hypermethylated and 5 were hypomethylated (Figure 3). The most divergent CpG was associated with the TNRC6A exon (p-value = 8.75E-08), a gene that encodes a protein involved in RNA-mediated post-transcriptional gene silencing (Table 3).

3.5 DISCUSSION

The role of DNAm in gene expression is taxonomically widespread, and its contribution to phenotypic variation has been documented in several mammal species (Cao et al., 2015; Ma et al., 2019; Varriale & Bernardi, 2006). Variations in DNAm have also been linked to body size (e.g. Rzehak et al., 2017; Cao et al., 2015; Renard et al., 2022), potentially as a result of differential methylation at genes involved in growth (e.g. Renard et al., 2022; Ma et al., 2019). Studies suggest a $r > 0.80$ in large independent data is indicative of validity in DNAm models (Anastasiadi & Piferrer, 2023; Horvath & Raj, 2018); here, we report the development and validation of a model that is predictive ($r = 0.88$, MAE = 1.31 cm) of hindfoot length in white-tailed deer, a species of North American large mammal that holds significant economic and cultural value to populations across its range. We also identified several CpG sites through EWAS that demonstrate significantly different methylation patterns associated with hindfoot length. These sites provide a starting point for further investigation into genes or pathways involved in body size, a polygenic trait in mammals with many underlying mechanisms (Posbergh & Huson, 2021; Makvandi-Nejad et al., 2012), including deer (Anderson et al. 2022).

3.5.1 Predictive ability of DNAm compared to age and sex

Model 3, which excluded sex, age class, and predicted age as predictor variables, demonstrated reduced error and higher accuracy in predicted hindfoot length when compared to models that included those three variables. This result is worth discussion as sex is often cited as a driver of variation in body size, and sexual size dimorphism has been reported in a large variety of mammals (McPherson & Chenoweth, 2012). Sexual size dimorphism has also specifically been reported in deer and other ungulates (McPherson & Chenoweth, 2012; Post et al., 1999) and deer show a clinal variation in size associated with latitude (Wolverton et al., 2009). Age is also a driver of body size in ungulate species (Sowande et al., 2010), with animals growing with age and eventually hitting an asymptote (Reiner et al., 2022). Although age and sex were excluded from this model, some of the extracted CpG sites demonstrated a positive correlation to age and sex; we therefore cannot concussively eliminate the influence of these variables, and further research needs to be done to clearly understand any pathways underlying this relationship. The stronger predictive ability of Model 3 compared to other models suggests that DNAm at sites correlated to age and sex (Supplemental Figure 1-3) may be more predictive of body size than biological age and sex. This could potentially be explained by the fact that DNAm integrates the effects of genetics, environment, life history, and disease, all of which can impact body size (Flores et al., 2013; Tangili et al., 2023; Jin & Liu, 2018; Sibly & Brown, 2007; Elayadeth-Meethal et al., 2018). In addition, the dynamic nature of DNAm allows it to change in response to varying environmental conditions (Flores et al., 2013), whereas chronological age and sex in mammals are impervious. Another explanation for the stronger predictive ability of Model 3, compared to models that include age as a predictive variable (e.g., Model 2), is that errors in age

prediction may negatively impact the accuracy of the associated models and cause them to perform worse than Model 3.

3.5.2 Applications to wildlife management

The ability to accurately predict hindfoot length of white-tailed deer can potentially be applied to wildlife management, as it allows for a non-invasive method of obtaining measures of body size; this analysis can be performed without handling animals and can be applied to cases where only tissue or non-invasive samples (i.e., fecal or hair) are available. Hindfoot is also correlated with body size metrics (Martin et al., 2013; Garel et al., 2010), and body size can act as an indicator of several factors that are important to consider when developing and applying interventions. For example, body size in white-tailed deer has been shown to inform breeding success, body size in mule deer has been shown to inform winter survival of fawns (Newbolt et al., 2016; Unsworth et al., 1999), and body size (as indicated by hindfoot length) has been linked to population density in roe deer (Zannèse et al., 2006). Related to this, Clements & Ozgul (2016) suggest that phenotypic traits such as body size can help predict population collapse; significant shifts in body size can act as an indicator that a population is approaching a tipping point. The identification of a small number of CpG sites where DNAm is predictive of hindfoot length (< 45) allows for the later development of targeted assays that can be implemented alongside other uses for DNAm analysis, such as assays aiming to predict age and sex (Czajka et al., 2024).

In conclusion, we demonstrate that DNA methylation is predictive of hindfoot length in white-tailed deer, and that epigenetic markers of hindfoot length may be stronger predictors of body size than age and sex when considered together. We also demonstrate the successful application hindfoot length and biological age estimation, suggesting that this approach can be

more widely applied in cases where phenotypic data is unavailable or traditional aging methods are insufficient. This is particularly useful in harvested species, as it can be applied to butchered and processed animals. This study offers novel insight on body size diversity in a free-ranging large mammal and may offer new avenues for investigating the complex pathways underlying phenotypic traits and variation in these traits.

TABLES AND FIGURES

Table 1. Basic sample information. *N* = total number of samples, number of females, number of hindfoot (HF) measurements, range of hindfoot length per age class, and range of predicted biological age. Hindfoot length is represented in centimeters, and age is represented in years.

Age Class	*N*	Number of females	Number of HF measurements	Range of HF length (cm)	Range of predicted bio. age
Fawn	56	31	46	29 - 49	2.28 - 3.59
Yearling	14	10	13	45 – 51.5	2.51 - 3.97
Adult	78	54	64	42.5 - 57	2.77 - 7.99

Table 2. Predictive variables included in each model. WTD CpGs refers to all 34,070 CpG sites found to align to the white-tailed deer genome. PCA 1 & 2 refers to component 1 and 2 from the principal component analysis performed on genotypes.

Predictive Variable	Model 1	Model 2	Model 3	Model 3.1	Model 3.2	Model 3.3	Model 3.4
Sex	Yes*	Yes*	No	Male Only*	Female Only*	Male Only*	Female Only*
Age Class	Yes	No	No	Adult + Yearling Only*	Adult + Yearling Only*	Fawn Only*	Fawn Only*
Predicted Age	No	Yes*	No	No	No	No	No
Capture Month	Yes*	Yes*	Yes*	Yes*	Yes*	Yes*	Yes*
WTD CpGs	Yes	Yes	Yes	Yes	Yes	Yes	Yes
PCA1 & 2	Yes*	Yes*	Yes*	Yes*	Yes*	Yes*	Yes*

** Indicates variables dropped by models (co-efficient penalized to zero).*

Table 3. Top significant CpGs from EWAS. Functions of each gene are abbreviated from Uniprot.

CGid	Gene & Region	Function	p	Direction
cg09461098	TNRC6A Exon	Plays a role in RNA-mediated gene silencing	8.75E-08	neg
cg10427592	ERCC1 Exon	Plays a role in DNA excision repair	1.48E-07	neg
cg10078834	PBX3 Intron	Transcriptional activator	1.83E-07	pos
cg01644993	AHDC1 Intron	Transcription factor required for proper patterning of the epidermis, plays a key role in early epithelial morphogenesis	2.38E-07	pos
cg19152887	BCL6 Intron	Transcriptional repressor	2.46E-07	neg
cg20483513	LOC100130987 Exon	Uncharacterized	3.97E-07	neg
cg22358400	TAOK3 fiveUTR	Involved in the G2/M transition DNA damage checkpoint as a regulator of the p38/MAPK14 stress-activated MAPK cascade.	5.69E-07	pos
cg27344974	SOX4 threeUTR	Transcriptional activator involved in skeletal myoblast differentiation	6.03E-07	pos
cg22802229	SPAG5-AS1 Exon	Uncharacterized	9.39E-07	neg

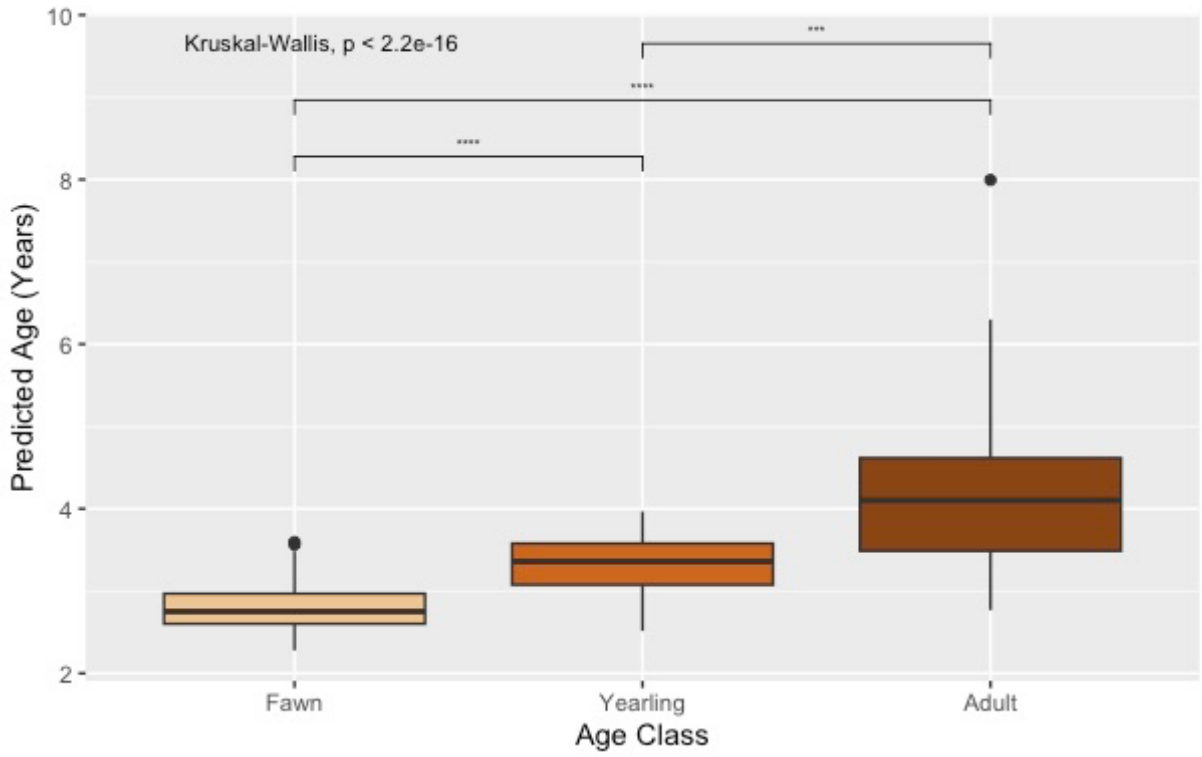


Figure 1. Biological age prediction of samples based on DNA methylation levels, using an established white-tailed deer epigenetic clock. P-value significance level for pairwise comparisons (Wilcoxon test) is represented by asterisks (*). P-value for the comparison between all three age classes (Kruskal-Wallis test) is reported.

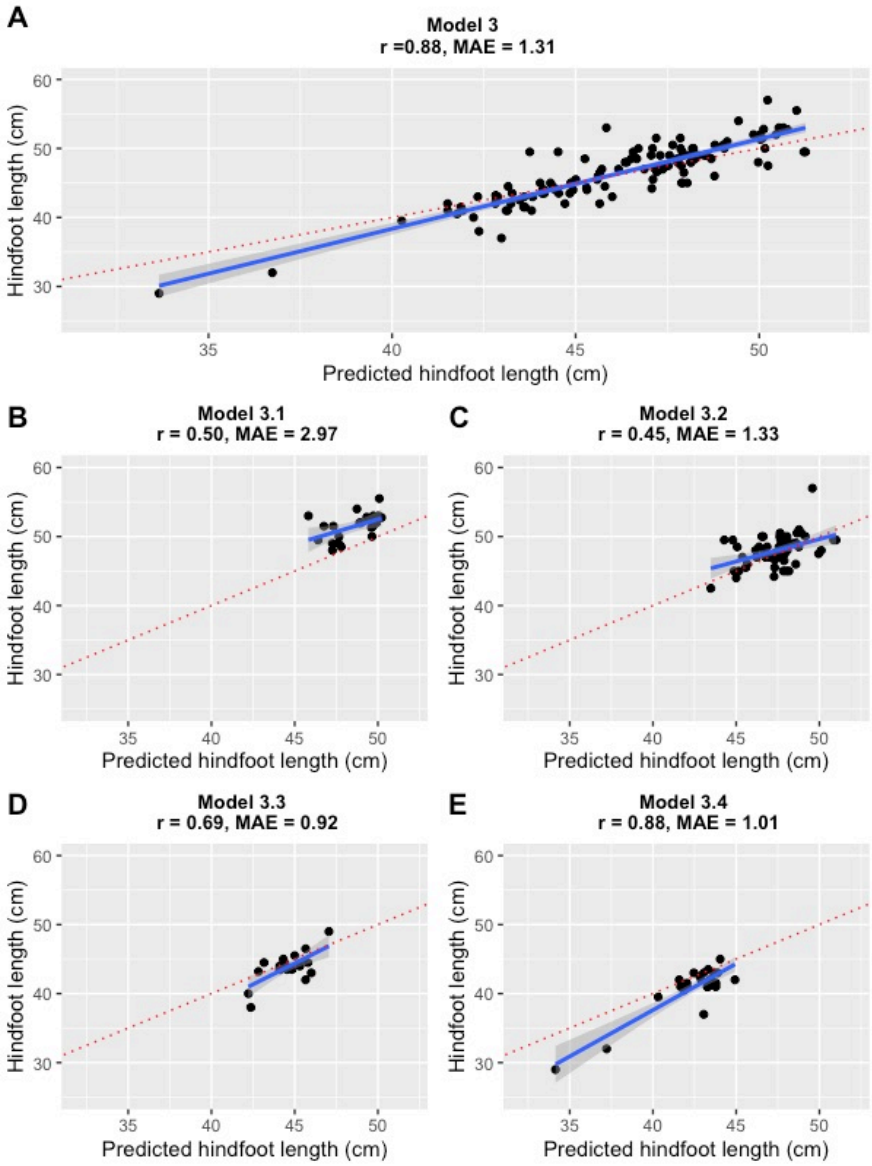


Figure 2. Cross-validation study of hindfoot length predictions for white-tailed deer based on **A** Model 3 and **B-E** subset sex- and age-specific models. DNAm-based prediction of hindfoot length (x-axis, in units of centimetres) versus measured hindfoot length (y-axis, in unit of centimetres). The solid line indicates the linear regression with standard error indicated by the shaded area, and the dashed line depicts the diagonal (y = x). Cor represents the correlation coefficient (*r*), and MAE represents the median absolute error (in unit of years).

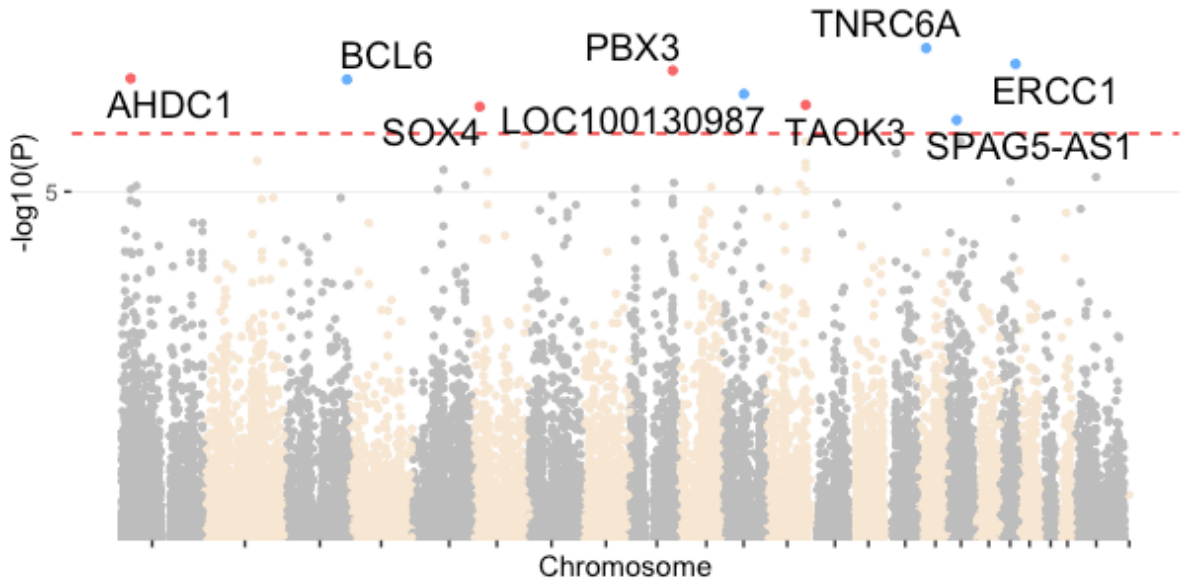


Figure 3. Manhattan plots of the Epigenome-wide association (EWAS) for hindfoot length in white-tailed deer. The coordinates are estimated based on the alignment of 34,070 Mammalian array probes to our white-tailed deer genome assembly. The direction of associations with p < 1.47e-6 (red dotted line) is highlighted by red (hypermethylated) and blue (hypomethylated) colours. Top 9 CpGs are indicated by their neighbouring genes.

CHAPTER 4: GENERAL DISCUSSION

I used genome-wide epigenetic techniques to investigate the mechanisms underlying phenotypic variation in several species of North American large mammals, namely white-tailed deer, black bear, and mountain goat. In accordance with predictions, I found support for a strong correlative relationship ($r > 0.95$) between DNA methylation (DNAm) and chronological age in all three species, and a reduced error in predicted ages when compared to previously published pan-mammalian epigenetic clocks. This is consistent with literature that suggests species-specific clocks improve the accuracy of age predictions (Peters et al., 2023).

There are a variety of avenues through which phenotypic data can be used to enhance wildlife management, and by effectively utilizing these data managers can enhance their ability to sustain wildlife populations in the face of environmental challenges. As demonstrated in this thesis, epigenetic clocks provide a reproducible and accurate tool for age prediction that has potential for use in wildlife management. Understanding age structure is key to maintaining managed populations at sustainable levels; by providing a non-invasive method of age estimation that can be implemented at a relatively large scale, epigenetics clocks can help managers assess population dynamics and trends or identify age-specific mortality rates. In terms of harvested populations, epigenetic clocks can inform hunting quotas and seasons based on population age structures, and can inform the need to limit or focus harvest on specific age classes and sexes (Milner et al., 2007).

In Chapter 3, I built a highly predictive model of white-tailed deer hindfoot length, a proxy of body size, using DNAm. Of note, while sex and age are clear drivers of variation in body size in deer and other ungulates (McPherson & Chenoweth, 2012; Post et al., 1999;

Sowande et al., 2010), my results suggest that DNAm is more predictive of body size than chronological age and biological sex. This could potentially be explained by the fact that DNAm integrates the effects of genetics, environment, life history, and disease, all of which can impact body size (Flores et al., 2013; Tangili et al., 2023; Jin & Liu, 2018; Sibly & Brown, 2007; Elayadeth-Meethal et al., 2018). In addition, the dynamic nature of DNAm allows it to change in response to varying environmental conditions (Flores et al., 2013), whereas chronological age and sex in mammals are impervious to external influence. In other words, methylated markers associated with sex and age capture more meaningful variation, and thus are statistically more informative than sex and age classifiers. By demonstrating a predictive relationship between hindfoot length and DNAm, we support the involvement of DNA methylation in body size variation and suggest new avenues for applying this relationship to wildlife management.

The ability to accurately predict hindfoot length of white-tailed deer can also be applied to wildlife management; body size can act as an indicator of several factors that are important to consider when developing and applying interventions, such as breeding success, winter survival, and population density (Newbolt et al., 2017; Unsworth et al., 1999; Zannèse et al., 2006). Accurate data on phenotypic traits such as body size can also improve the accuracy of predictions when incorporated into population models, particularly when predicting population collapse (Clements & Ozgul, 2016). Overall, DNA methylation can be used to monitor, or augment monitoring, of wildlife populations by providing accurate phenotypic data. This data can be used to assess the need for management interventions and to track their effectiveness.

Overall, by performing epigenome-wide associated studies (EWAS), I was able to identify individual sites across all three species' genomes where significantly different methylation levels are associated with age class and sex, as well as hindfoot length in white-

tailed deer. These results indicate that coarse phenotypic data can be obtained using targeted analyses, negating the need for genome-wide analysis and increasing accessibility while decreasing cost. My results indicate that DNA methylation is a useful tool for predicting phenotypic traits in samples where observable phenotypic data is unavailable. This tool is particularly useful for commonly harvested species, as it can be applied to butchered and processed animals, and species where age identification is typically dependent on external features such horns (e.g. in the case of a mountain goat with broken horns).

The epigenetic clocks developed in this thesis exhibit similar patterns in error as those found in other mammals (e.g., Caulton et al., 2021; Robeck et al., 2023); however, the residuals tend to increase with age, particularly in bears and mountain goats, suggesting a reduced accuracy in older individuals. A possible explanation for this is errors in the chronological ages used in our model, as a decline in the accuracy of traditional aging methods with age is common in all three study species (Stevens & Houston, 1989; Harshyne et al., 1998; Storm et al., 2014; Foley et al., 2021). To address this issue, future studies could include individuals aged to a higher degree of confidence, such as those in longitudinal studies (Boertje et al., 2019).

We also identified several CpG sites through EWAS that exhibit variations in methylation patterns linked to hindfoot length. These sites can serve as a starting point for future investigation into the genes or pathways involved in body size, a polygenic trait in mammals with numerous underlying mechanisms (Posbergh & Huson, 2021; Makvandi-Nejad et al., 2012). Future studies could perform a gene enrichment analysis of these differentially methylated CpGs to investigate related pathways and biological systems.

In conclusion, this thesis demonstrates the use of DNA methylation as a marker of phenotype in three iconic North American large mammals. Through this research, we add to our

understanding of DNAm's role in phenotypic expression, and we introduce new avenues for applying this relationship to wildlife management with the potential to contribute to wildlife monitoring by providing easily obtainable representations of age, sex, and body size in managed populations. Incorporating the epigenetic perspective into wildlife management and research can lead to more effective strategies, helping ensure the preservation of wildlife populations in a rapidly changing world.

REFERENCES

Anastasiadi, D., Esteve-Codina, A., & Piferrer, F. (2018). Consistent inverse correlation between DNA methylation of the first intron and gene expression across tissues and species. *Epigenetics & Chromatin*, *11*(1), 37. https://doi.org/10.1186/s13072-018-0205-1

Anastasiadi, D., & Piferrer, F. (2023). Bioinformatic analysis for age prediction using epigenetic clocks: Application to fisheries management and conservation biology. *Frontiers in Marine Science*, *10*. https://doi.org/10.3389/fmars.2023.1096909

Anderson, S. J., Côté, S. D., Richard, J. H., & Shafer, A. B. A. (2022). Genomic architecture of phenotypic extremes in a wild cervid. *BMC Genomics*, *23*(1), 126. https://doi.org/10.1186/s12864-022-08333-x

Arneson, A., Haghani, A., Thompson, M. J., Pellegrini, M., Kwon, S. B., Vu, H., Maciejewski, E., Yao, M., Li, C. Z., Lu, A. T., Morselli, M., Rubbi, L., Barnes, B., Hansen, K. D., Zhou, W., Breeze, C. E., Ernst, J., & Horvath, S. (2022). A mammalian methylation array for profiling methylation levels at conserved sequences. *Nature Communications*, *13*(1), Article 1. https://doi.org/10.1038/s41467-022-28355-z

Beckmann, J. P., & Berger, J. (2003). Rapid ecological and behavioural changes in carnivores: The responses of black bears (Ursus americanus) to altered food. *Journal of Zoology*, *261*(2), 207–212. https://doi.org/10.1017/S0952836903004126

Beier, L. R., Lewis, S. B., Flynn, R. W., Pendleton, G., & Schumacher, T. V. (2005). From the Field: A single-catch snare to collect brown bear hair for genetic mark-recapture studies. *Wildlife Society Bulletin*, *33*(2), 766–773. https://doi.org/10.2193/0091-7648(2005)33[766:FTFASS]2.0.CO;2

Bendixen, K. K., Mindegaard, M., Epistolio, S., Dazio, G., Marchi, F., Spina, P., Arnspang, E. C., Soerensen, M., Christensen, U. B., Frattini, M., & Petersen, R. K. (2023). A qPCR technology for direct quantification of methylation in untreated DNA. *Nature Communications*, *14*(1), Article 1. https://doi.org/10.1038/s41467-023-40873-y

Blackburn, T. M., & Hawkins, B. A. (2004). Bergmann's rule and the mammal fauna of northern North America. *Ecography*, *27*(6), 715–724. https://doi.org/10.1111/j.0906-7590.2004.03999.x

Bobak, C. A., Abhimanyu, Natarajan, H., Gandhi, T., Grimm, S. L., Nishiguchi, T., Koster, K., Longlax, S. C., Dlamini, Q., Kahari, J., Mtetwa, G., Cirillo, J. D., O'Malley, J., Hill, J. E., Coarfa, C., & DiNardo, A. R. (2022). Increased DNA methylation, cellular senescence and premature epigenetic aging in guinea pigs and humans with tuberculosis. *Aging (Albany NY)*, *14*(5), 2174–2193. https://doi.org/10.18632/aging.203936

Boertje, R. D., Frye, G. G., & Young Jr., D. D. (2019). Lifetime, known-age moose reproduction in a nutritionally stressed population. *The Journal of Wildlife Management*, *83*(3), 610–626. https://doi.org/10.1002/jwmg.21613

Bollati, V., & Baccarelli, A. (2010). Environmental epigenetics. *Heredity*, *105*(1), 105–112. https://doi.org/10.1038/hdy.2010.2

Brans, K. I., Jansen, M., Vanoverbeke, J., Tüzün, N., Stoks, R., & De Meester, L. (2017). The heat is on: Genetic adaptation to urbanization mediated by thermal tolerance and body size. *Global Change Biology*, *23*(12), 5218–5227. https://doi.org/10.1111/gcb.13784

Brearley, G., Rhodes, J., Bradley, A., Baxter, G., Seabrook, L., Lunney, D., Liu, Y., & McAlpine, C. (2013). Wildlife disease prevalence in human-modified landscapes. *Biological Reviews*, *88*(2), 427–442. https://doi.org/10.1111/brv.12009

Brisotto, G., di Gennaro, A., Damiano, V., Armellin, M., Perin, T., Maestro, R., & Santarosa, M. (2015). An improved sequencing-based strategy to estimate locus-specific DNA methylation. *BMC Cancer*, *15*(1), 639. https://doi.org/10.1186/s12885-015-1646-6

Cafaro, P., Hansson, P., & Götmark, F. (2022). Overpopulation is a major cause of biodiversity loss and smaller human populations are necessary to preserve what is left. *Biological Conservation*, *272*, 109646. https://doi.org/10.1016/j.biocon.2022.109646

Cao, J., Wei, C., Liu, D., Wang, H., Wu, M., Xie, Z., Capellini, T. D., Zhang, L., Zhao, F., Li, L., Zhong, T., Wang, L., Lu, J., Liu, R., Zhang, S., Du, Y., Zhang, H., & Du, L. (2015). DNA methylation Landscape of body size variation in sheep. *Scientific Reports*, *5*(1), 13950. https://doi.org/10.1038/srep13950

Cardillo, M., Mace, G. M., Jones, K. E., Bielby, J., Bininda-Emonds, O. R. P., Sechrest, W., Orme, C. D. L., & Purvis, A. (2005). Multiple Causes of High Extinction Risk in Large Mammal Species. *Science*, *309*(5738), 1239–1241. https://doi.org/10.1126/science.1116030

Caulton, A., Dodds, K. G., McRae, K. M., Couldrey, C., Horvath, S., & Clarke, S. M. (2021). Development of Epigenetic Clocks for Key Ruminant Species. *Genes*, *13*(1), 96. https://doi.org/10.3390/genes13010096

Chinnadurai, S. K., Strahl-Heldreth, D., Fiorello, C. V., & Harms, C. A. (2016). Best-practice guidelines for field-based surgery and anesthesia of free-ranging wildlife. I. Anesthesia and analgesia. *Journal of Wildlife Diseases*, *52*(2s), S14–S27. https://doi.org/10.7589/52.2S.S14

Clark, J. D., Beckmann, J. P., Boyce, M. S., Leopold, B. D., & Pelton, M. R. (2021). *American Black Bear (Ursus americanus)*. 122–138.

Clements, C. F., & Ozgul, A. (2016). Including trait-based early warning signals helps predict population collapse. *Nature Communications*, *7*(1), 10984. https://doi.org/10.1038/ncomms10984

Clover, M. R. (1954). A portable deer trap and catch-net. *California Fish and Game*, *40*(4), 367–373.

Cossette, M.-L., Stewart, D. T., Haghani, A., Zoller, J. A., Shafer, A. B. A., & Horvath, S. (2023). Epigenetics and island-mainland divergence in an insectivorous small mammal. *Molecular Ecology*, *32*(1), 152–166. https://doi.org/10.1111/mec.16735

Côté, S. D., & Festa-Bianchet, M. (2001). Birthdate, mass and survival in mountain goat kids: Effects of maternal characteristics and forage quality. *Oecologia*, *127*(2), 230–238. https://doi.org/10.1007/s004420000584

Côté, S., & Festa-Bianchet, M. (2003). *Wild Mammals of North America: Biology, Management, Conservation* (pp. 1061–1075).

Crist, E., Mora, C., & Engelman, R. (2017). The interaction of human population, food production, and biodiversity protection. *Science*, *356*(6335), 260–264. https://doi.org/10.1126/science.aal2011

Cullingham, C. I., Nakada, S. M., Merrill, E. H., Bollinger, T. K., Pybus, M. J., & Coltman, D. W. (2011). Multiscale population genetic analysis of mule deer (Odocoileus hemionus hemionus) in western Canada sheds new light on the spread of chronic wasting disease. *Canadian Journal of Zoology*, *89*(2), 134–147. https://doi.org/10.1139/Z10-104

Czajka, N., Northrup, J. M., Jones, M. J., & Shafer, A. B. A. (2024). Epigenetic clocks, sex markers and age-class diagnostics in three harvested large mammals. *Molecular Ecology Resources*, *24*(5), e13956. https://doi.org/10.1111/1755-0998.13956

Darlington, S., Ladle, A., Burton, A. C., Volpe, J. P., & Fisher, J. T. (2022). Cumulative effects of human footprint, natural features and predation risk best predict seasonal resource selection by white-tailed deer. Scientific Reports, 12(1), 1072. https://doi.org/10.1038/s41598-022-05018-z

Davis, K. L., Silverman, E. D., Sussman, A. L., Wilson, R. R., & Zipkin, E. F. (2022). Errors in aerial survey count data: Identifying pitfalls and solutions. *Ecology and Evolution*, *12*(3), e8733. https://doi.org/10.1002/ece3.8733

Delgiudice, G. D., Fieberg, J., Riggs, M. R., Powell, M. C., & Pan, W. (2006). A Long-Term Age-Specific Survival Analysis of Female White-Tailed Deer. The Journal of Wildlife Management, 70(6), 1556–1568. https://doi.org/10.2193/0022-541X(2006)70[1556:ALASAO]2.0.CO;2

Dhar, G. A., Saha, S., Mitra, P., & Nag Chaudhuri, R. (2021). DNA methylation and regulation of gene expression: Guardian of our health. *The Nucleus*, *64*(3), 259–270. https://doi.org/10.1007/s13237-021-00367-y

Elayadeth-Meethal, M., Thazhathu Veettil, A., Maloney, S. K., Hawkins, N., Misselbrook, T. H., Sejian, V., Rivero, M. J., & Lee, M. R. F. (2018). Size does matter: Parallel evolution of adaptive thermal tolerance and body size facilitates adaptation to climate change in domestic cattle. *Ecology and Evolution*, *8*(21), 10608–10620. https://doi.org/10.1002/ece3.4550

Fleckhaus, J., & Schneider, P. M. (2020). Novel multiplex strategy for DNA methylation-based age prediction from small amounts of DNA via Pyrosequencing. *Forensic Science International: Genetics*, *44*, 102189. https://doi.org/10.1016/j.fsigen.2019.102189

Flores, K. B., Wolschin, F., & Amdam, G. V. (2013). The Role of Methylation of DNA in Environmental Adaptation. *Integrative and Comparative Biology*, *53*(2), 359–372. https://doi.org/10.1093/icb/ict019

Foley, A. M., Lewis, J. S., Cortez, O., Hellickson, M. W., Hewitt, D. G., DeYoung, R. W., DeYoung, C. A., Schnupp, M. J., Foley, A. M., Lewis, J. S., Cortez, O., Hellickson, M. W., Hewitt, D. G., DeYoung, R. W., DeYoung, C. A., & Schnupp, M. J. (2021). Accuracies and biases of ageing white-tailed deer in semiarid environments. *Wildlife Research*, *49*(3), 237–249. https://doi.org/10.1071/WR21050

Fortin, J.-P., Triche, T. J., Jr, & Hansen, K. D. (2017). Preprocessing, normalization and integration of the Illumina HumanMethylationEPIC array with minfi. *Bioinformatics*, *33*(4), 558–560. https://doi.org/10.1093/bioinformatics/btw691

Friedman, J. H., Hastie, T., & Tibshirani, R. (2010). Regularization Paths for Generalized Linear Models via Coordinate Descent. *Journal of Statistical Software*, *33*, 1–22. https://doi.org/10.18637/jss.v033.i01

Gaidatzis, D., Lerch, A., Hahne, F., & Stadler, M. B. (2015). QuasR: Quantification and annotation of short reads in R. *Bioinformatics (Oxford, England)*, *31*(7), 1130–1132. https://doi.org/10.1093/bioinformatics/btu781

Gallego-Bartolomé, J. (2020). DNA methylation in plants: Mechanisms and tools for targeted manipulation. *New Phytologist*, *227*(1), 38–44. https://doi.org/10.1111/nph.16529

Garel, M., Gaillard, J.-M., Chevrier, T., Michallet, J., Delorme, D., & Van Laere, G. (2010). Testing Reliability of Body Size Measurements Using Hind Foot Length in Roe Deer. *The Journal of Wildlife Management*, *74*(6), 1382–1386. https://doi.org/10.1111/j.1937-2817.2010.tb01261.x

Gasaway, W. C., Harkness, D. B., & Rausch, R. A. (1978). Accuracy of Moose Age Determinations from Incisor Cementum Layers. *The Journal of Wildlife Management*, *42*(3), 558–563. https://doi.org/10.2307/3800818

Gibney, E. R., & Nolan, C. M. (2010). Epigenetics and gene expression. *Heredity*, *105*(1), 4–13. https://doi.org/10.1038/hdy.2010.54

Goldstein, M. I., Poe, A. J., Cooper, E., Youkey, D., Brown, B. A., & McDonald, T. L. (2005). Mountain goat response to helicopter overflights in Alaska. *Wildlife Society Bulletin*, *33*(2), 688–699. https://doi.org/10.2193/0091-7648(2005)33[688:MGRTHO]2.0.CO;2

Haghani, A., Li, C. Z., Robeck, T. R., Zhang, J., Lu, A. T., Ablaeva, J., Acosta-Rodríguez, V. A., Adams, D. M., Alagaili, A. N., Almunia, J., Aloysius, A., Amor, N. M. S., Ardehali, R., Arneson, A., Baker, C. S., Banks, G., Belov, K., Bennett, N. C., Black, P., … Horvath, S. (2023). DNA methylation networks underlying mammalian traits. *Science*, *381*(6658), eabq5693. https://doi.org/10.1126/science.abq5693

Han, Y., Eipel, M., Franzen, J., Sakk, V., Dethmers-Ausema, B., Yndriago, L., Izeta, A., de Haan, G., Geiger, H., & Wagner, W. (n.d.). Epigenetic age-predictor for mice based on three CpG sites. *eLife*, *7*, e37462. https://doi.org/10.7554/eLife.37462

Hao, T., Guo, J., Liu, J., Wang, J., Liu, Z., Cheng, X., Li, J., Ren, J., Li, Z., Yan, J., & Zhang, G. (2021). Predicting human age by detecting DNA methylation status in hair. *Electrophoresis*, *42*(11), 1255–1261. https://doi.org/10.1002/elps.202000349

Harris, R. B., & Metzgar, L. H. (1987). Harvest Age Structures as Indicators of Decline in Small Populations of Grizzly Bears. *Bears: Their Biology and Management*, *7*, 109–116. https://doi.org/10.2307/3872616

Harshyne, W. A., Diefenbach, D. R., Alt, G. L., & Matson, G. M. (1998). Analysis of Error from Cementum-Annuli Age Estimates of Known-Age Pennsylvania Black Bears. *The Journal of Wildlife Management*, *62*(4), 1281–1291. https://doi.org/10.2307/3801992

Hayes, B. J., Nguyen, L. T., Forutan, M., Engle, B. N., Lamb, H. J., Copley, J. P., Randhawa, I. A. S., & Ross, E. M. (2021). An Epigenetic Aging Clock for Cattle Using Portable Sequencing Technology. *Frontiers in Genetics*, *12*. https://www.frontiersin.org/articles/10.3389/fgene.2021.760450

He, Q., & Silliman, B. R. (2019). Climate Change, Human Impacts, and Coastal Ecosystems in the Anthropocene. *Current Biology*, *29*(19), R1021–R1035. https://doi.org/10.1016/j.cub.2019.08.042

Hecht, L. (2021). The importance of considering age when quantifying wild animals' welfare. *Biological Reviews*, *96*(6), 2602–2616. https://doi.org/10.1111/brv.12769

Hetem, R. S., Fuller, A., Maloney, S. K., & Mitchell, D. (2014). Responses of large mammals to climate change. *Temperature*, *1*(2), 115–127. https://doi.org/10.4161/temp.29651

Hewitt, D. G. (2015). Hunters and the conservation and management of white-tailed deer (Odocoileus virginianus). *International Journal of Environmental Studies*, *72*(5), 839–849. https://doi.org/10.1080/00207233.2015.1073473

Hohenlohe, P. A., Funk, W. C., & Rajora, O. P. (2021). Population genomics for wildlife conservation and management. *Molecular Ecology*, *30*(1), 62–82. https://doi.org/10.1111/mec.15720

Honey, M., Johnson, J., Menke, C., Cruz, A. R., Karwacki, J., & Durham, W. H. (2016). The comparative economic value of bear viewing and bear hunting in the Great Bear Rainforest. *Journal of Ecotourism*, *15*(3), 199–240. https://doi.org/10.1080/14724049.2016.1142554

Horvath, S., & Raj, K. (2018). DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nature Reviews Genetics*, *19*(6), 371–384. https://doi.org/10.1038/s41576-018-0004-3

Hristienko, H., & McDonald, J. E. (2007). Going into the 21st Century: A Perspective on Trends and Controversies in the Management of the American Black Bear. *Ursus*, *18*(1), 72–88.

Jin, Z., & Liu, Y. (2018). DNA methylation in human diseases. *Genes & Diseases*, *5*(1), 1–8. https://doi.org/10.1016/j.gendis.2018.01.002

Johnson, R. C., Nelson, G. W., Troyer, J. L., Lautenberger, J. A., Kessing, B. D., Winkler, C. A., & O'Brien, S. J. (2010). Accounting for multiple comparisons in a genome-wide association study (GWAS). *BMC Genomics*, *11*(1), 724. https://doi.org/10.1186/1471-2164-11-724

Jones, M. J., Goodman, S. J., & Kobor, M. S. (2015). DNA methylation and healthy human aging. *Aging Cell*, *14*(6), 924–932. https://doi.org/10.1111/acel.12349

Jung, M., & Pfeifer, G. P. (2015). Aging and DNA methylation. *BMC Biology*, *13*(1), 7. https://doi.org/10.1186/s12915-015-0118-4

Kelly, M. (2019). Adaptation to climate change through genetic accommodation and assimilation of plastic phenotypes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *374*(1768), 20180176. https://doi.org/10.1098/rstb.2018.0176

Kendall, K. C., Stetz, J. B., Roon, D. A., Waits, L. P., Boulanger, J. B., & Paetkau, D. (2008). Grizzly Bear Density in Glacier National Park, Montana. *The Journal of Wildlife Management*, *72*(8), 1693–1705. https://doi.org/10.2193/2008-007

Lacher, T. E., Jr., Davidson, A. D., Fleming, T. H., Gómez-Ruiz, E. P., McCracken, G. F., Owen-Smith, N., Peres, C. A., & Vander Wall, S. B. (2019). The functional roles of

mammals in ecosystems. *Journal of Mammalogy*, *100*(3), 942–964. https://doi.org/10.1093/jmammal/gyy183

Laliberte, A. S., & Ripple, W. J. (2004). Range Contractions of North American Carnivores and Ungulates. BioScience, 54(2), 123–138. https://doi.org/10.1641/0006-3568(2004)054[0123:RCONAC]2.0.CO;2

Larison, B., Pinho, G. M., Haghani, A., Zoller, J. A., Li, C. Z., Finno, C. J., Farrell, C., Kaelin, C. B., Barsh, G. S., Wooding, B., Robeck, T. R., Maddox, D., Pellegrini, M., & Horvath, S. (2021). Epigenetic models developed for plains zebras predict age in domestic horses and endangered equids. *Communications Biology*, *4*(1), Article 1. https://doi.org/10.1038/s42003-021-02935-z

Le Clercq, L.-S., Kotzé, A., Grobler, J. P., & Dalton, D. L. (2023). Biological clocks as age estimation markers in animals: A systematic review and meta-analysis. *Biological Reviews*, *98*(6), 1972–2011. https://doi.org/10.1111/brv.12992

Leek, J. T., Johnson, W. E., Parker, H. S., Jaffe, A. E., & Storey, J. D. (2012). The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics*, *28*(6), 882–883. https://doi.org/10.1093/bioinformatics/bts034

Lenart, P., & Bienertová-Vašků, J. (2017). Keeping up with the Red Queen: The pace of aging as an adaptation. *Biogerontology*, *18*(4), 693–709. https://doi.org/10.1007/s10522-016-9674-4

Leroux, S. J., Wiersma, Y. F., & Vander Wal, E. (2020). Herbivore Impacts on Carbon Cycling in Boreal Forests. *Trends in Ecology & Evolution*, *35*(11), 1001–1010. https://doi.org/10.1016/j.tree.2020.07.009

Liu, C., Xu, L., Li, W., Jie, M., Xue, W., & Yu, W. (2021). Multiple Biomarker-Combined Screening for Colorectal Cancer Based on Bisulfate Conversion-Free Detection of Fecal

DNA Methylation. *BioMed Research International*, *2021*, e1479748. https://doi.org/10.1155/2021/1479748

Liu, Z., He, C., & Wu, J. (2016). The Relationship between Habitat Loss and Fragmentation during Urbanization: An Empirical Evaluation from 16 World Cities. *PLOS ONE*, *11*(4), e0154613. https://doi.org/10.1371/journal.pone.0154613

Loewe, L., & Hill, W. G. (2010). The population genetics of mutations: Good, bad and indifferent. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1544), 1153–1167. https://doi.org/10.1098/rstb.2009.0317

Lu, A. T., Fei, Z., Haghani, A., Robeck, T. R., Zoller, J. A., Li, C. Z., Lowe, R., Yan, Q., Zhang, J., Vu, H., Ablaeva, J., Acosta-Rodriguez, V. A., Adams, D. M., Almunia, J., Aloysius, A., Ardehali, R., Arneson, A., Baker, C. S., Banks, G., … Horvath, S. (2023). Universal DNA methylation age across mammalian tissues. *Nature Aging*, 1–23. https://doi.org/10.1038/s43587-023-00462-6

Lyko, F. (2018). The DNA methyltransferase family: A versatile toolkit for epigenetic regulation. *Nature Reviews Genetics*, *19*(2), Article 2. https://doi.org/10.1038/nrg.2017.80

Ma, X., Jia, C., Chu, M., Fu, D., Lei, Q., Ding, X., Wu, X., Guo, X., Pei, J., Bao, P., Yan, P., & Liang, C. (2019). Transcriptome and DNA Methylation Analyses of the Molecular Mechanisms Underlying with Longissimus dorsi Muscles at Different Stages of Development in the Polled Yak. *Genes*, *10*(12), Article 12. https://doi.org/10.3390/genes10120970

Makvandi-Nejad, S., Hoffman, G. E., Allen, J. J., Chu, E., Gu, E., Chandler, A. M., Loredo, A. I., Bellone, R. R., Mezey, J. G., Brooks, S. A., & Sutter, N. B. (2012). Four Loci Explain

83% of Size Variation in the Horse. *PLOS ONE*, *7*(7), e39929. https://doi.org/10.1371/journal.pone.0039929

Marantz, S. A., Long, J. A., Webb, S. L., Gee, K. L., Little, A. R., & Demarais, S. (2016). Impacts of human hunting on spatial behavior of white-tailed deer (Odocoileus virginianus). Canadian Journal of Zoology, 94(12), 853–861. https://doi.org/10.1139/cjz-2016-0125

Martchenko, D., White, K. S., & Shafer, A. B. A. (2022). Long-term data reveal effects of climate, road access, and latitude on mountain goat horn size. *The Journal of Wildlife Management*, *86*(3), e22195. https://doi.org/10.1002/jwmg.22195

Martin, J. G. A., Festa-Bianchet, M., Côté, S. D., & Blumstein, D. T. (2013). Detecting between-individual differences in hind-foot length in populations of wild mammals. *Canadian Journal of Zoology*, *91*(3), 118–123. https://doi.org/10.1139/cjz-2012-0210

Martins, A. C. R. (2011). Change and Aging Senescence as an Adaptation. PLOS ONE, 6(9), e24328. https://doi.org/10.1371/journal.pone.0024328

McCance, E. C., Decker, D. J., Colturi, A. M., Baydack, R. K., Siemer, W. F., Curtis, P. D., & Eason, T. (2017). Importance of Urban Wildlife Management in the United States and Canada. *Mammal Study*, *42*(1), 1–16. https://doi.org/10.3106/041.042.0108

McLean, C., Gluckman, P., & Sheppard, A. (2012). Phenotypic diversity and epigenomic variation – The utility of mass spectrometric analysis of DNA methylation. *Journal of Proteomics*, *75*(12), 3400–3409. https://doi.org/10.1016/j.jprot.2012.01.036

McPherson, F. J., & Chenoweth, P. J. (2012). Mammalian sexual dimorphism. *Animal Reproduction Science*, *131*(3), 109–122. https://doi.org/10.1016/j.anireprosci.2012.02.007

McShea, W. J. (2012). Ecology and management of white-tailed deer in a changing world. *Annals of the New York Academy of Sciences*, *1249*(1), 45–56. https://doi.org/10.1111/j.1749-6632.2011.06376.x

McVean, G. (2009). A Genealogical Interpretation of Principal Components Analysis. *PLoS Genetics*, *5*(10), e1000686. https://doi.org/10.1371/journal.pgen.1000686

Milner, J. M., Nilsen, E. B., & Andreassen, H. P. (2007). Demographic Side Effects of Selective Hunting in Ungulates and Carnivores. *Conservation Biology*, *21*(1), 36–47. https://doi.org/10.1111/j.1523-1739.2006.00591.x

Moore, L. D., Le, T., & Fan, G. (2013). DNA Methylation and Its Basic Function. *Neuropsychopharmacology*, *38*(1), Article 1. https://doi.org/10.1038/npp.2012.112

Morrison, J. C., Sechrest, W., Dinerstein, E., Wilcove, D. S., & Lamoreux, J. F. (2007). Persistence of Large Mammal Faunas as Indicators of Global Human Impacts. *Journal of Mammalogy*, *88*(6), 1363–1380. https://doi.org/10.1644/06-MAMM-A-124R2.1

Nachtomy, O., Shavit, A., & Yakhini, Z. (2007). Gene expression and the concept of the phenotype. *Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences*, *38*(1), 238–254. https://doi.org/10.1016/j.shpsc.2006.12.014

Nelson, S. L., Carr, P. C., McBride, A., & Aubry, L. M. (2024). Predictors of female American black bear body mass in an anthropogenic landscape. *The Journal of Wildlife Management*, *88*(4), e22577. https://doi.org/10.1002/jwmg.22577

Newbolt, C. H., Acker, P. K., Neuman, T. J., Hoffman, S. I., Ditchkoff, S. S., & Steury, T. D. (2017). Factors influencing reproductive success in male white-tailed deer. *The Journal of Wildlife Management*, *81*(2), 206–217. https://doi.org/10.1002/jwmg.21191

Nicholson, T. E., Mayer, K. A., Staedler, M. M., Gagné, T. O., Murray, M. J., Young, M. A., Tomoleoni, J. A., Tinker, M. T., & Van Houtan, K. S. (2020). Robust age estimation of southern sea otters from multiple morphometrics. *Ecology and Evolution*, *10*(16), 8592–8609. https://doi.org/10.1002/ece3.6493

Pacht, E., Murdoch, B., & McKay, S. (2021). Examining the extent of environmental contributions toward DNA methylation and phenotypic variation. *Animal Frontiers*, *11*(6), 83–89. https://doi.org/10.1093/af/vfab056

Parlee, B., Ahkimnachie, K., Cunningham, H., Jordan, M., & Goddard, E. (2021). "It's important to know about this"—Risk communication and the impacts of chronic wasting disease on indigenous food systems in Western Canada. *Environmental Science & Policy*, *123*, 190–201. https://doi.org/10.1016/j.envsci.2021.05.012

Peters, K. J., Gerber, L., Scheu, L., Cicciarella, R., Zoller, J. A., Fei, Z., Horvath, S., Allen, S. J., King, S. L., Connor, R. C., Rollins, L. A., & Krützen, M. (2023). An epigenetic DNA methylation clock for age estimates in Indo-Pacific bottlenose dolphins (Tursiops aduncus). *Evolutionary Applications*, *16*(1), 126–133. https://doi.org/10.1111/eva.13516

Pettorelli, N., Pelletier, F., Hardenberg, A. von, Festa-Bianchet, M., & Côté, S. D. (2007). Early Onset of Vegetation Growth Vs. Rapid Green-up: Impacts on Juvenile Mountain Ungulates. *Ecology*, *88*(2), 381–390. https://doi.org/10.1890/06-0875

Polanowski, A. M., Robbins, J., Chandler, D., & Jarman, S. N. (2014). Epigenetic estimation of age in humpback whales. *Molecular Ecology Resources*, *14*(5), 976–987. https://doi.org/10.1111/1755-0998.12247

Posbergh, C. J., & Huson, H. J. (2021). All sheeps and sizes: A genetic investigation of mature body size across sheep breeds reveals a polygenic nature. *Animal Genetics*, *52*(1), 99–107. https://doi.org/10.1111/age.13016

Post, E., Langvatn, R., Forchhammer, M. C., & Stenseth, N. Chr. (1999). Environmental variation shapes sexual dimorphism in red deer. *Proceedings of the National Academy of Sciences*, *96*(8), 4467–4471. https://doi.org/10.1073/pnas.96.8.4467

Reiner, R., Zedrosser, A., Zeiler, H., Hackländer, K., & Corlatti, L. (2022). Habitat and climate shape growth patterns in a mountain ungulate. *Ecology and Evolution*, *12*(3), e8650. https://doi.org/10.1002/ece3.8650

Renard, T., Gueydan, C., & Aron, S. (2022). DNA methylation and expression of the egfr gene are associated with worker size in monomorphic ants. *Scientific Reports*, *12*(1), 21228. https://doi.org/10.1038/s41598-022-25675-4

Reynolds-Hogland, M., Ramsey, A. B., Muench, C., Pilgrim, K. L., Engkjer, C., Erba, G., & Ramsey, P. W. (2022). Integrating video and genetic data to estimate annual age-structured apparent survival of American black bears. Population Ecology, 64(4), 300–322. https://doi.org/10.1002/1438-390X.12122

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research*, *43*(7), e47. https://doi.org/10.1093/nar/gkv007

Robeck, T. R., Fei, Z., Lu, A. T., Haghani, A., Jourdain, E., Zoller, J. A., Li, C. Z., Steinman, K. J., DiRocco, S., Schmitt, T., Osborn, S., Van Bonn, B., Katsumata, E., Mergl, J., Almunia, J., Rodriguez, M., Haulena, M., Dold, C., & Horvath, S. (2021). Multi-species and multi-

tissue methylation clocks for age estimation in toothed whales and dolphins. *Communications Biology*, *4*(1), Article 1. https://doi.org/10.1038/s42003-021-02179-x

Robeck, T. R., Haghani, A., Fei, Z., Lindemann, D. M., Russell, J., Herrick, K. E. S., Montano, G., Steinman, K. J., Katsumata, E., Zoller, J. A., & Horvath, S. (2023). Multi-tissue DNA methylation aging clocks for sea lions, walruses and seals. *Communications Biology*, *6*(1), Article 1. https://doi.org/10.1038/s42003-023-04734-0

Rosenfeld, C. S. (2010). Animal Models to Study Environmental Epigenetics1. *Biology of Reproduction*, *82*(3), 473–488. https://doi.org/10.1095/biolreprod.109.080952

Rzehak, P., Covic, M., Saffery, R., Reischl, E., Wahl, S., Grote, V., Weber, M., Xhonneux, A., Langhendries, J.-P., Ferre, N., Closa-Monasterolo, R., Escribano, J., Verduci, E., Riva, E., Socha, P., Gruszfeld, D., & Koletzko, B. (2017). DNA-Methylation and Body Composition in Preschool Children: Epigenome-Wide-Analysis in the European Childhood Obesity Project (CHOP)-Study. *Scientific Reports*, *7*(1), 14349. https://doi.org/10.1038/s41598-017-13099-4

Samuel, C. (1990). *The Chilkat Dancing Blanket*. University of Oklahoma Press.

Sato, A., Hiura, H., Okae, H., Miyauchi, N., Abe, Y., Utsunomiya, T., Yaegashi, N., & Arima, T. (2011). Assessing loss of imprint methylation in sperm from subfertile men using novel methylation polymerase chain reaction Luminex analysis. *Fertility and Sterility*, *95*(1), 129–134, 134.e1-4. https://doi.org/10.1016/j.fertnstert.2010.06.076

Scheick, B. K., & McCown, W. (2014). Geographic distribution of American black bears in North America. *Ursus*, *25*(1), 24–33. https://doi.org/10.2192/URSUS-D-12-00020.1

Schuster, R. C., Wein, E. E., Dickson, C., & Chan, H. M. (2011). Importance of traditional foods for the food security of two First Nations communities in the Yukon, Canada. *International Journal of Circumpolar Health*, *70*(3), 286–300. https://doi.org/10.3402/ijch.v70i3.17833

Sibly, R. M., & Brown, J. H. (2007). Effects of body size and lifestyle on evolution of mammal life histories. *Proceedings of the National Academy of Sciences*, *104*(45), 17707–17712. https://doi.org/10.1073/pnas.0707725104

Sinclair, A. R. E. (2003). The Role of Mammals as Ecosystem Landscapers. *Alces: A Journal Devoted to the Biology and Management of Moose*, *39*, 161–176.

Sinke, L., Cats, D., & Heijmans, B. T. (2021). Omixer: Multivariate and reproducible sample randomization to proactively counter batch effects in omics studies. *Bioinformatics (Oxford, England)*, *37*(18), 3051–3052. https://doi.org/10.1093/bioinformatics/btab159

Smith, B. L. (1988). Criteria for Determining Age and Sex of American Mountain Goats in the Field. *Journal of Mammalogy*, *69*(2), 395–402. https://doi.org/10.2307/1381400

Smith, W. P. (1991). Odocoileus virginianus. *Mammalian Species*, *388*, 1–13. https://doi.org/10.2307/3504281

Solberg, E. J., Saether, B.-E., Strand, O., & Loison, A. (1999). Dynamics of a harvested moose population in a variable environment. *Journal of Animal Ecology*, *68*(1), 186–204. https://doi.org/10.1046/j.1365-2656.1999.00275.x

Sowande, O. S., Oyewale, B. F., & Iyasere, O. S. (2010). Age- and sex-dependent regression models for predicting the live weight of West African Dwarf goat from body measurements. *Tropical Animal Health and Production*, *42*(5), 969–975. https://doi.org/10.1007/s11250-009-9515-4

Stevens, V., & Houston, D. B. (1989). Reliability of Age Determination of Mountain Goats. *Wildlife Society Bulletin (1973-2006)*, *17*(1), 72–74.

St-Louis, A., Hamel, S., Mainguy, J., & Côté, S. D. (2013). Factors influencing the reaction of mountain goats towards all-terrain vehicles. *The Journal of Wildlife Management*, *77*(3), 599–605. https://doi.org/10.1002/jwmg.488

Storm, D. J., Samuel, M. D., Rolley, R. E., Beissel, T., Richards, B. J., & Van Deelen, T. R. (2014). Estimating ages of white-tailed deer: Age and sex patterns of error using tooth wear-and-replacement and consistency of cementum annuli. *Wildlife Society Bulletin*, *38*(4), 849–856. https://doi.org/10.1002/wsb.457

Sucharzewski, S. (2024). Assessing the Drivers of White-Tailed Deer (Odocoileus virginianus) Migration: Exploring Changing Predation Risk and Food Availability in an Ontario Population. ProQuest Dissertations & Theses.

Tangili, M., Slettenhaar, A. J., Sudyka, J., Dugdale, H. L., Pen, I., Palsbøll, P. J., & Verhulst, S. (2023). DNA methylation markers of age(ing) in non-model animals. *Molecular Ecology*, *n/a*(n/a). https://doi.org/10.1111/mec.17065

Teschendorff, A. E., West, J., & Beck, S. (2013). Age-associated epigenetic drift: Implications, and a case of epigenetic thrift? *Human Molecular Genetics*, *22*(R1), R7–R15. https://doi.org/10.1093/hmg/ddt375

Tryland, M., Beckmen, K. B., Burek-Huntington, K. A., Breines, E. M., & Klein, J. (2018). Orf virus infection in Alaskan mountain goats, Dall's sheep, muskoxen, caribou and Sitka black-tailed deer. *Acta Veterinaria Scandinavica*, *60*(1), 12. https://doi.org/10.1186/s13028-018-0366-8

Udevitz, M. S., & Ballachey, B. E. (1998). Estimating Survival Rates with Age-Structure Data. *The Journal of Wildlife Management*, *62*(2), 779–792. https://doi.org/10.2307/3802355

Unnikrishnan, A., Freeman, W. M., Jackson, J., Wren, J. D., Porter, H., & Richardson, A. (2019). The role of DNA methylation in epigenetics of aging. *Pharmacology & Therapeutics*, *195*, 172–185. https://doi.org/10.1016/j.pharmthera.2018.11.001

Unnikrishnan, A., Hadad, N., Masser, D. R., Jackson, J., Freeman, W. M., & Richardson, A. (2018). Revisiting the genomic hypomethylation hypothesis of aging. *Annals of the New York Academy of Sciences*, *1418*(1), 69–79. https://doi.org/10.1111/nyas.13533

Unsworth, J. W., Pac, D. F., White, G. C., & Bartmann, R. M. (1999). Mule Deer Survival in Colorado, Idaho, and Montana. *The Journal of Wildlife Management*, *63*(1), 315–326. https://doi.org/10.2307/3802515

Varriale, A., & Bernardi, G. (2006). DNA methylation and body temperature in fishes. *Gene*, *385*, 111–121. https://doi.org/10.1016/j.gene.2006.05.031

Vayro, J. V., Vandermale, E. A., & Mason, C. W. (2023). 'It's a people problem, not a goat problem.' Mitigating human–mountain goat interactions in a Canadian Provincial Park. *Wildlife Research*, *50*(11), 911–926. https://doi.org/10.1071/WR22005

Veiberg, V., Nilsen, E. B., Rolandsen, C. M., Heim, M., Andersen, R., Holmstrøm, F., Meisingset, E. L., & Solberg, E. J. (2020). The accuracy and precision of age determination by dental cementum annuli in four northern cervids. *European Journal of Wildlife Research*, *66*(6), 91. https://doi.org/10.1007/s10344-020-01431-9

Waller, D. M., & Alverson, W. S. (1997). The White-Tailed Deer: A Keystone Herbivore. *Wildlife Society Bulletin (1973-2006)*, *25*(2), 217–226.

Wan, X., Jiang, G., Yan, C., He, F., Wen, R., Gu, J., Li, X., Ma, J., Stenseth, N. Chr., & Zhang, Z. (2019). Historical records reveal the distinctive associations of human disturbance and extreme climate change with local extinction of mammals. *Proceedings of the National Academy of Sciences*, *116*(38), 19001–19008. https://doi.org/10.1073/pnas.1818019116

White, K. S., & Gregovich, D. P. (2017). Mountain goat resource selection in relation to mining-related disturbance. *Wildlife Biology*, *2017*(1), wlb.00277. https://doi.org/10.2981/wlb.00277

White, K. S., Pendleton, G. W., Crowley, D., Griese, H. J., Hundertmark, K. J., Mcdonough, T., Nichols, L., Robus, M., Smith, C. A., & Schoen, J. W. (2011). Mountain goat survival in coastal Alaska: Effects of age, sex, and climate. The Journal of Wildlife Management, 75(8), 1731–1744. https://doi.org/10.1002/jwmg.238

Wilkinson, G. S., Adams, D. M., Haghani, A., Lu, A. T., Zoller, J., Breeze, C. E., Arnold, B. D., Ball, H. C., Carter, G. G., Cooper, L. N., Dechmann, D. K. N., Devanna, P., Fasel, N. J., Galazyuk, A. V., Günther, L., Hurme, E., Jones, G., Knörnschild, M., Lattenkamp, E. Z., … Horvath, S. (2021). DNA methylation predicts age and provides insight into exceptional longevity of bats. *Nature Communications*, *12*(1), Article 1. https://doi.org/10.1038/s41467-021-21900-2

Willi, Y., Kristensen, T. N., Sgrò, C. M., Weeks, A. R., Ørsted, M., & Hoffmann, A. A. (2022). Conservation genetics as a management tool: The five best-supported paradigms to assist the management of threatened species. *Proceedings of the National Academy of Sciences*, *119*(1), e2105076119. https://doi.org/10.1073/pnas.2105076119

Wilmers, C. C., & Getz, W. M. (2005). Gray Wolves as Climate Change Buffers in Yellowstone. *PLOS Biology*, *3*(4), e92. https://doi.org/10.1371/journal.pbio.0030092

Wolverton, S., Huston, M. A., Kennedy, J. H., Cagle, K., & Cornelius, J. D. (2009). Conformation to Bergmann's Rule in White-Tailed Deer Can Be Explained by Food Availability. *The American Midland Naturalist*, *162*(2), 403–417.

Woodward, G., Ebenman, B., Emmerson, M., Montoya, J. M., Olesen, J. M., Valido, A., & Warren, P. H. (2005). Body size in ecological networks. *Trends in Ecology & Evolution*, *20*(7), 402–409. https://doi.org/10.1016/j.tree.2005.04.005

Yagi, S., Hirabayashi, K., Sato, S., Li, W., Takahashi, Y., Hirakawa, T., Wu, G., Hattori, N., Hattori, N., Ohgane, J., Tanaka, S., Liu, X. S., & Shiota, K. (2008). DNA methylation profile of tissue-dependent and differentially methylated regions (T-DMRs) in mouse promoter regions demonstrating tissue-specific gene expression. *Genome Research*, *18*(12), 1969–1978. https://doi.org/10.1101/gr.074070.107

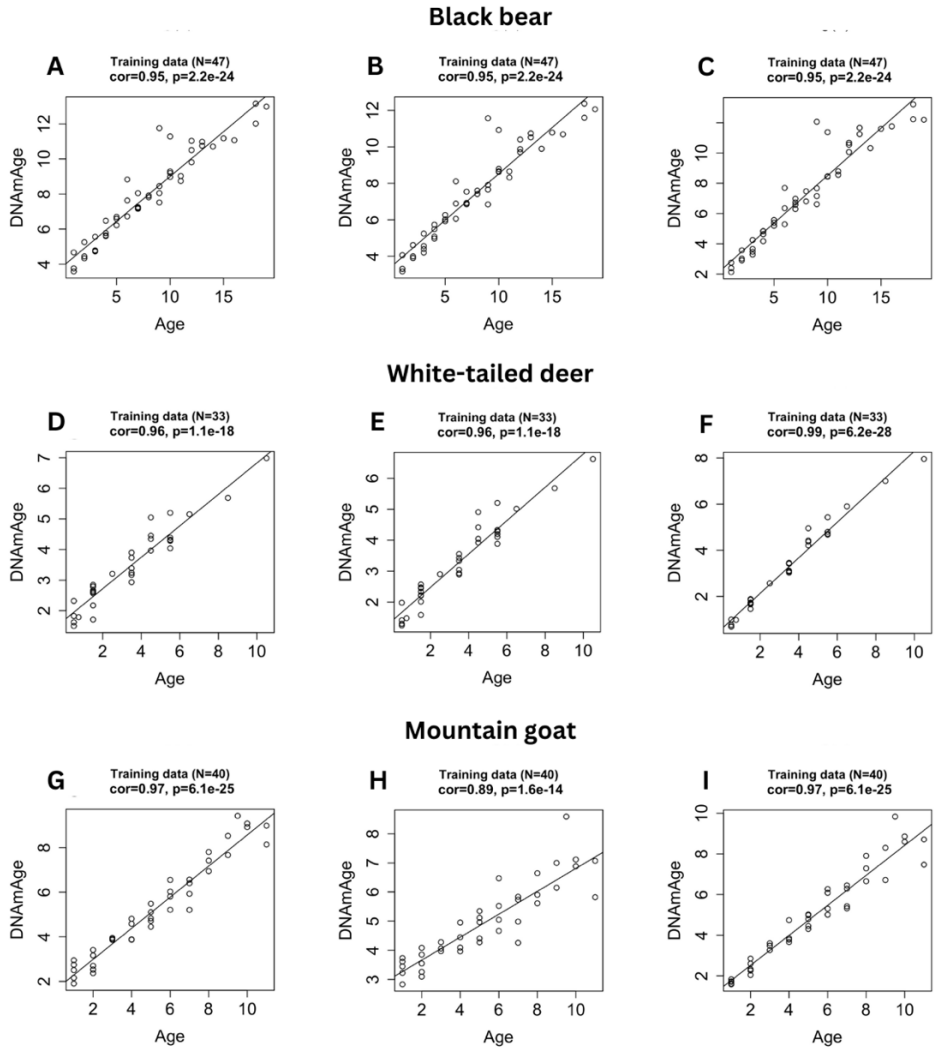
Zannas, A. S., Arloth, J., Carrillo-Roa, T., Iurato, S., Röh, S., Ressler, K. J., Nemeroff, C. B., Smith, A. K., Bradley, B., Heim, C., Menke, A., Lange, J. F., Brückl, T., Ising, M., Wray, N. R., Erhardt, A., Binder, E. B., & Mehta, D. (2015). Lifetime stress accelerates epigenetic aging in an urban, African American cohort: Relevance of glucocorticoid signaling. *Genome Biology*, *16*(1), 266. https://doi.org/10.1186/s13059-015-0828-5

Zannèse, A., Baïsse, A., Gaillard, J.-M., Hewison, A. J. M., Saint-Hilaire, K., Toïgo, C., Van Laere, G., & Morellet, N. (2006). Hind Foot Length: An Indicator for Monitoring Roe Deer Populations at a Landscape Scale. *Wildlife Society Bulletin*, *34*(2), 351–358. https://doi.org/10.2193/0091-7648(2006)34[351:HFLAIF]2.0.CO;2

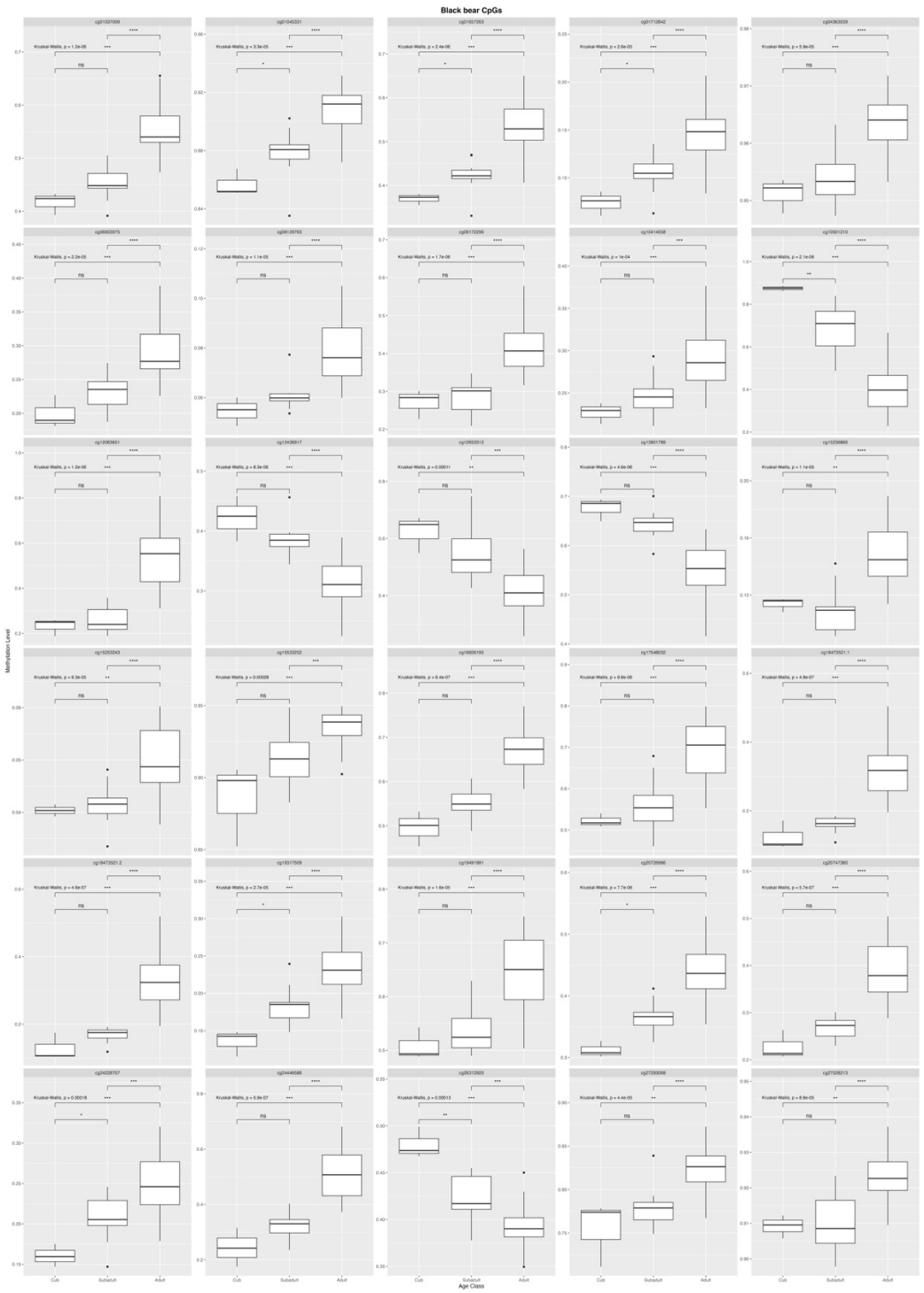
Zheng, S., Hu, J., Ma, Z., Lindenmayer, D., & Liu, J. (2023). Increases in intraspecific body size variation are common among North American mammals and birds between 1880 and 2020. *Nature Ecology & Evolution*, *7*(3), 347–354. https://doi.org/10.1038/s41559-022-01967-w

APPENDIX A: CHAPTER 2 SUPPORTING INFORMATION

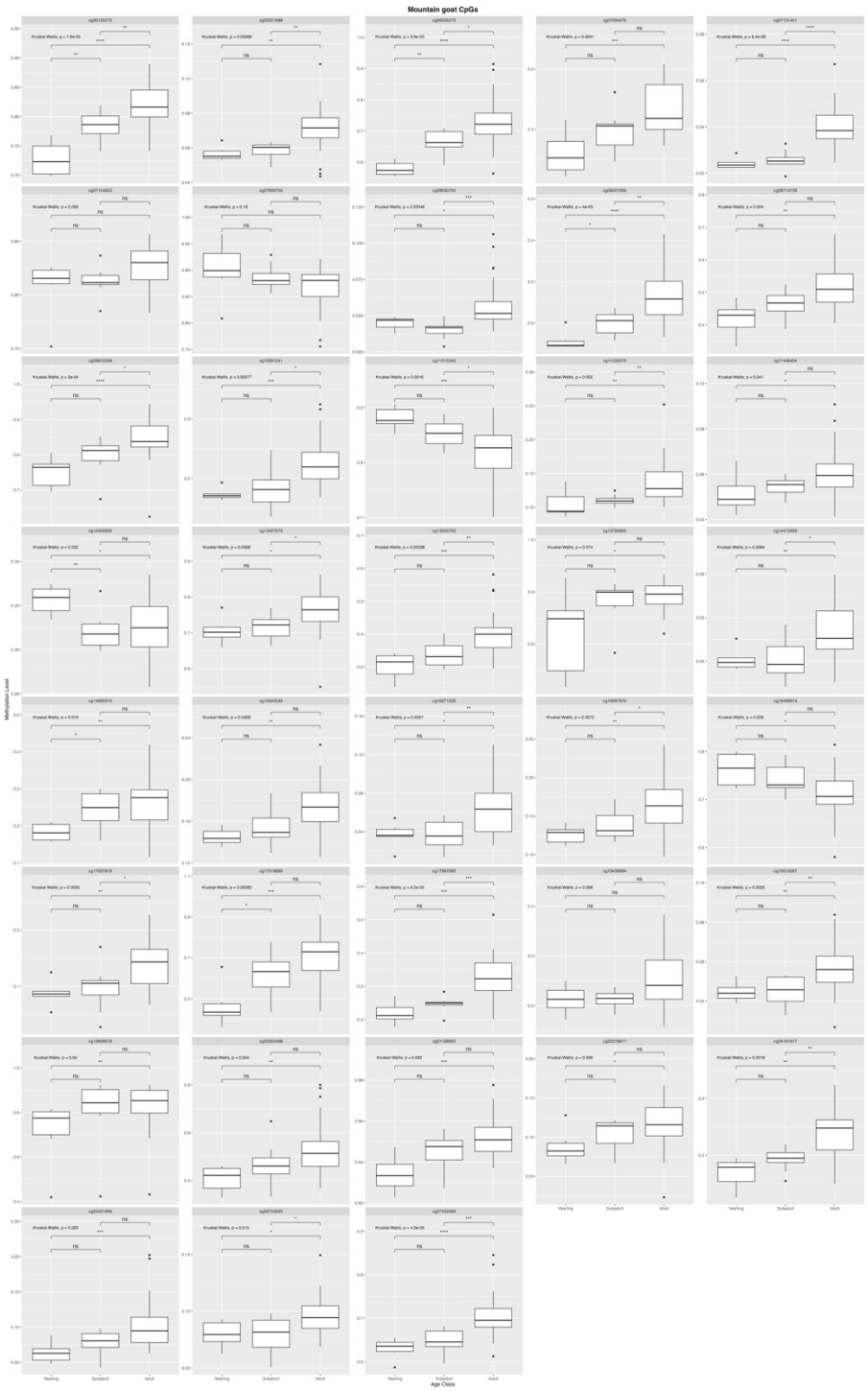
CHAPTER 2 SUPPLEMENTAL FIGURES



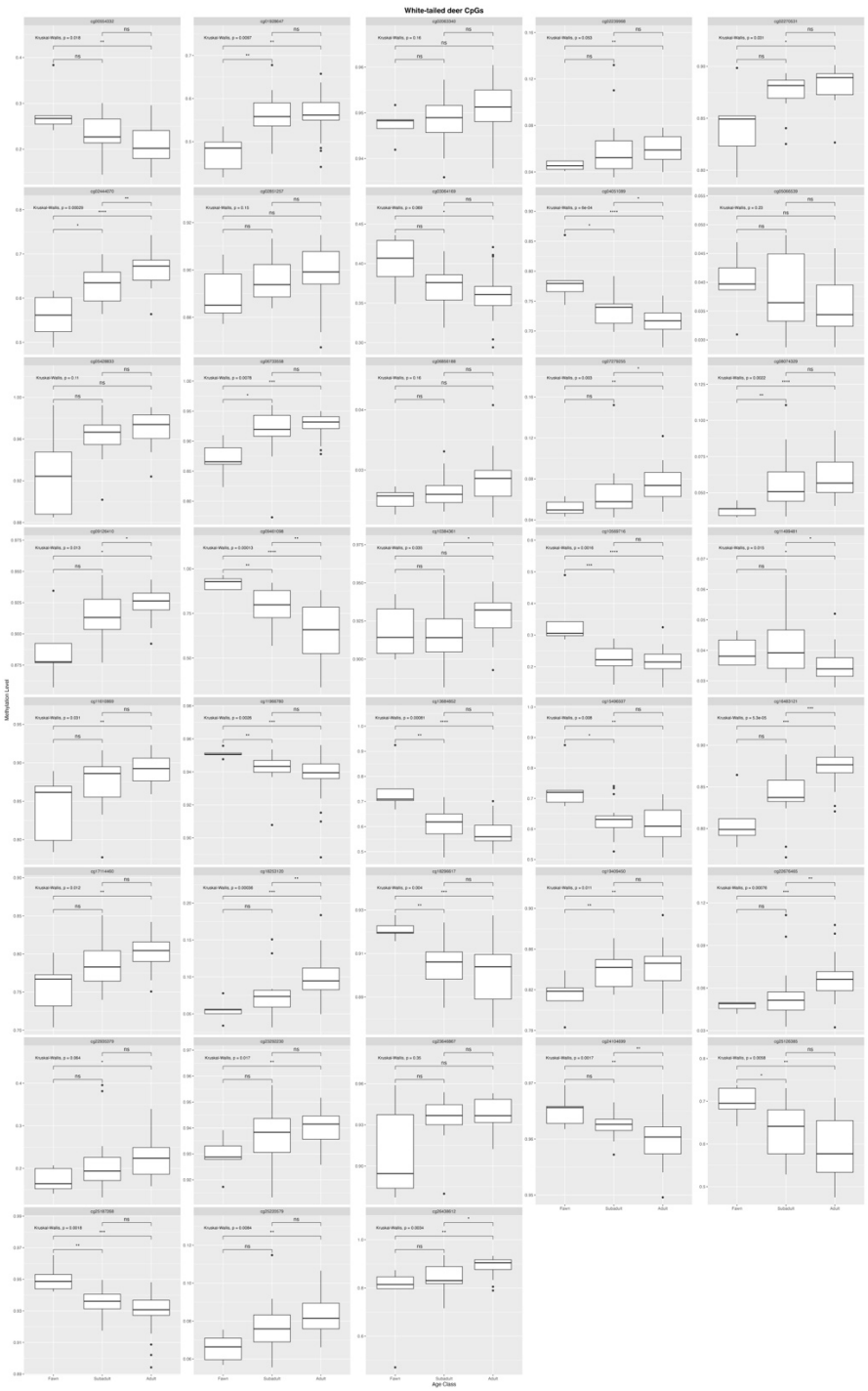
Supplemental Figure S1. Leave-one-out cross-validation study of species-specific epigenetic clocks for **A-C** black bear, **D-F** white-tailed deer, and **G-I** mountain goat. No age transformation **(A, D, G)**, square-root transformed chronological ages **(B, E, H),** and log-transformed chronological ages (**C, F, I**). DNAm age prediction (y-axis, in units of years) versus estimated chronological age (x-axis, in unit of years). The solid line indicates the linear regression of epigenetic age, and the dashed line depicts the diagonal (y = x). Cor represents the correlation coefficient (*r*), and "p" reports the calculated p-value.



Supplemental Figure S2. Boxplot of methylation level across age classes at all significant CpGs in black bear. P-value significance level for the comparison between pairs of classes (Wilcoxon test) is represented by asterisks (*). P-value for the comparison between all three classes (Kruskal-Wallis test) is reported for each CpG.



Supplemental Figure S3. Boxplot of methylation level across age classes at all significant CpGs in mountain goat. P-value significance level for the comparison between pairs of classes (Wilcoxon test) is represented by asterisks (*). P-value for the comparison between all three classes (Kruskal-Wallis test) is reported for each CpG.



Supplemental Figure S4. Boxplot of methylation level across age classes at all significant CpGs in white-tailed deer. P-value significance level for the comparison between pairs of classes (Wilcoxon test) is represented by asterisks (*). P-value for the comparison between all three classes (Kruskal-Wallis test) is reported for each CpG.

APPENDIX B: CHAPTER 3 SUPPORTING INFORMATION

CHAPTER 3 SUPPLEMENTAL TABLES

Supplemental Table 1. CpGs and associated beta values from a species-specific epigenetic clock for white-tailed deer.

CGID	Beta
cg05428833	1.36117645
cg22935379	0.37166311
cg00554332	-0.6299279
cg01928647	0.01106674
cg02063340	0.04776388
cg02239968	1.05881411
cg02270531	0.02290675
cg02444070	0.22612136
cg02851257	0.9018096
cg03064169	-0.0192843
cg04051089	-0.6827343
cg05066539	-1.5819306
cg06733558	0.71226978
cg06856188	2.70905465
cg07279255	2.62246995
cg08074329	6.85675947
cg09126410	2.50235158
cg09461098	-0.0060298
cg10384361	2.76445235
cg10569716	-0.6704699
cg11499481	-0.2044642
cg11610869	0.28797998
cg11966780	-7.5463623
cg13684852	-0.2024593
cg15496507	-0.3349292
cg16483121	0.5059376
cg17114460	0.15156859
cg18253120	0.07056795
cg18296617	-0.3933837
cg19409450	0.60708448
cg22676465	3.0755611
cg23292230	1.14838527
cg23646867	0.12447655

cg24104699	-1.324515
cg25126385	-0.1503408
cg25187268	-0.8806314
cg25220579	0.29499315
cg26438612	0.13154

Supplemental Table 2. All Model Output variables and Coefficients

CGid	Model 1	Model 2	Model 3	Model 3.1	Model 3.2	Model 3.3	Model 3.4
(Intercept)	3.220	3.093	3.382	2.926	3.093	3.382	3.197
Fawn	-0.096	NA	NA	NA	NA	NA	NA
cg01137610	-0.022	-0.017	-0.033	NA	-0.017	-0.033	-0.026
cg01241934	-0.006	NA	NA	NA	NA	NA	NA
cg02294059	-0.086	-0.239	-0.235	-0.230	-0.239	-0.235	-0.237
cg06162430	0.133	0.206	0.240	0.164	0.206	0.240	0.218
cg06443336	0.016	NA	NA	NA	NA	NA	NA
cg06644235	0.034	0.045	0.048	0.042	0.045	0.048	0.044
cg07863286	0.017	NA	NA	NA	NA	NA	NA
cg08575708	0.144	0.124	0.095	0.140	0.124	0.095	0.115
cg10078834	0.175	0.426	0.434	0.411	0.426	0.434	0.429
cg10739156	-0.090	-0.046	-0.041	-0.062	-0.046	-0.041	-0.044
cg10864340	-0.176	NA	NA	NA	NA	NA	NA
cg12619862	0.082	NA	NA	NA	NA	NA	NA
cg14878567	0.035	0.174	0.233	0.114	0.174	0.233	0.194
cg14988225	-0.085	-0.065	-0.058	-0.072	-0.065	-0.058	-0.065
cg16029938	-0.001	-0.019	-0.029	NA	-0.019	-0.029	-0.024
cg16269759	0.104	0.048	0.058	0.052	0.048	0.058	0.051
cg26360022	0.303	0.294	0.278	0.322	0.294	0.278	0.285
cg26768041	0.073	0.102	0.087	0.107	0.102	0.087	0.096
cg27501104	-0.248	-0.306	-0.348	-0.266	-0.306	-0.348	-0.321
cg05698015	NA	0.094	0.251	0.139	0.094	0.251	0.141
cg00794219	NA	0.013	0.027	NA	0.013	0.027	0.017
cg01128516	NA	0.141	0.115	0.100	0.141	0.115	0.134
cg01324452	NA	0.011	0.106	NA	0.011	0.106	0.044
cg01593408	NA	-0.026	-0.065	-0.007	-0.026	-0.065	-0.037

cg01644993	NA	0.036	0.014	0.008	0.036	0.014	0.032
cg01669013	NA	-0.103	-0.123	-0.073	-0.103	-0.123	-0.112
cg01799731	NA	0.061	0.062	0.016	0.061	0.062	0.063
cg01946401.2	NA	-0.035	NA	-0.019	-0.035	NA	-0.024
cg03683496	NA	-0.081	-0.106	-0.031	-0.081	-0.106	-0.090
cg07187097	NA	-0.165	-0.552	NA	-0.165	-0.552	-0.297
cg08013479	NA	-0.011	-0.017	NA	-0.011	-0.017	-0.015
cg09040561	NA	-0.068	-0.065	-0.055	-0.068	-0.065	-0.068
cg10243630	NA	0.000	NA	0.072	0.000	NA	NA
cg11025714	NA	-0.018	-0.009	-0.033	-0.018	-0.009	-0.013
cg12186717	NA	-0.167	-0.133	-0.184	-0.167	-0.133	-0.150
cg12412302	NA	0.142	0.162	0.108	0.142	0.162	0.151
cg12505751	NA	0.002	0.062	NA	0.002	0.062	0.023
cg12959926	NA	-0.002	-0.046	NA	-0.002	-0.046	-0.018
cg16574861	NA	0.002	0.066	NA	0.002	0.066	0.022
cg17475770	NA	-0.094	-0.129	-0.050	-0.094	-0.129	-0.110
cg17691933	NA	0.000	-0.020	NA	0.000	-0.020	-0.006
cg18266944	NA	-0.037	-0.034	-0.053	-0.037	-0.034	-0.037
cg19152887	NA	-0.062	-0.067	-0.054	-0.062	-0.067	-0.067
cg22802229	NA	-0.062	-0.040	-0.076	-0.062	-0.040	-0.056
cg24455383	NA	0.077	0.065	0.081	0.077	0.065	0.072
cg27344974	NA	0.139	0.122	0.142	0.139	0.122	0.134
cg06732973	NA	NA	0.012	0.005	NA	0.012	0.002
cg09269677	NA	NA	0.034	NA	NA	0.034	NA
cg17279431	NA	NA	-0.001	NA	NA	-0.001	NA
cg20043130	NA	NA	-0.089	NA	NA	-0.089	-0.032
cg25583823	NA	NA	-0.069	NA	NA	-0.069	-0.015

Supplemental Table 3. Hindfoot length predictions from all models. All values are reported in centimetres (cm).

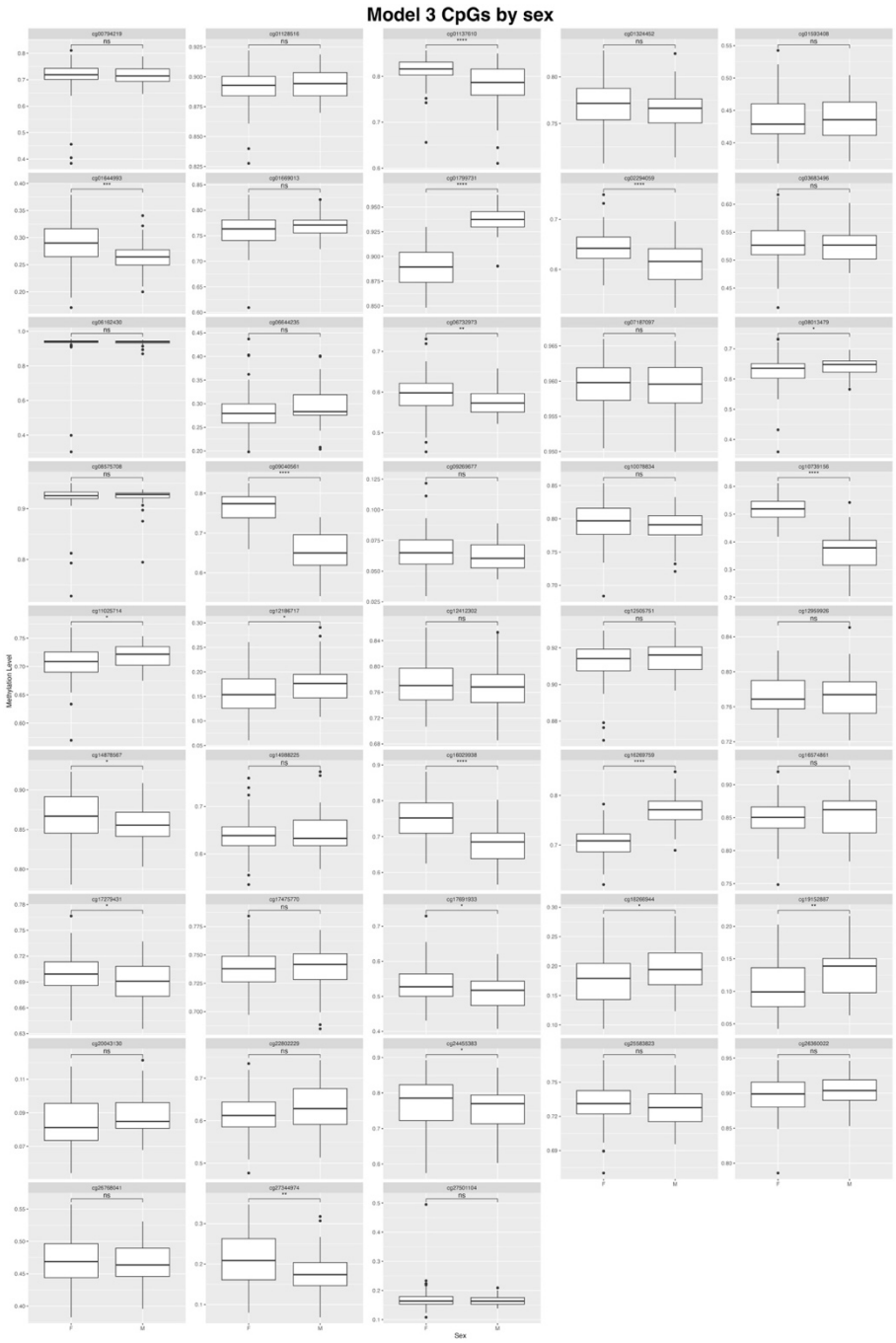
Sample ID	Hindfoot Length	Model 1	Model 2	Model 3	Model 3.1	Model 3.2	Model 3.3	Model 3.4
AD068	41	46.85	43.35	43.11	NA	NA	NA	43.26
AD012	50	48.43	48.05	48.21	NA	48.05	NA	NA
AD009	41.5	47.27	43.80	43.62	NA	NA	NA	43.74
TI11	47	48.38	47.02	47.16	NA	47.02	NA	NA
AD052	52.8	49.71	49.75	50.12	49.33	NA	NA	NA
TI10	52.75	49.82	50.55	50.77	50.22	NA	NA	NA
TI03	41.5	47.23	43.88	43.58	NA	NA	NA	43.78
AD005	41	46.61	41.72	41.52	NA	NA	NA	41.66
AD082	43	46.92	43.16	42.88	NA	NA	NA	43.06
AD081	50.5	48.24	48.65	48.79	NA	48.65	NA	NA
TI02	47	46.84	45.37	45.71	NA	45.37	NA	NA
AD070	52	49.90	49.41	49.85	48.99	NA	NA	NA
TI01	45.5	48.20	47.31	47.11	NA	47.31	NA	NA
TI07	46.5	47.79	47.88	47.95	NA	47.88	NA	NA
AD062	44	47.65	45.00	44.93	NA	45.00	NA	NA
AD084	49	48.96	48.41	48.51	NA	48.41	NA	NA
AD060	45	48.02	48.17	48.04	NA	48.17	NA	NA
AD076	38	46.62	42.73	42.37	NA	NA	42.37	NA
AD071	45	47.32	44.87	44.87	NA	44.87	NA	NA
AD067	47.5	46.96	47.35	47.44	NA	47.35	NA	NA
TI05	40.5	46.45	41.92	41.77	NA	NA	NA	41.87
AD065	51.5	49.65	47.56	47.85	47.32	NA	NA	NA
TI08	47	46.83	46.77	46.87	NA	46.77	NA	NA
AD064	52	49.84	49.49	50.02	48.91	NA	NA	NA
AD069	45	47.04	45.16	44.89	NA	45.16	NA	NA
AD002	41.5	46.20	42.12	41.87	NA	NA	NA	42.03
AD055	45	47.83	48.01	47.92	NA	48.01	NA	NA
AD018	44.3	49.17	45.63	45.30	NA	NA	45.30	NA
TI06	47.5	47.98	47.91	47.88	NA	47.91	NA	NA
AD013	48	48.30	48.02	48.13	NA	48.02	NA	NA
AD059	51	47.95	48.78	49.13	NA	48.78	NA	NA
AD080	48.5	47.73	46.38	46.51	NA	46.38	NA	NA
AD016	48	47.41	47.82	47.80	NA	47.82	NA	NA
TI09	49	48.48	47.88	47.94	47.65	NA	NA	NA
AD003	48	47.04	46.17	46.39	NA	46.17	NA	NA

AD057	32	41.64	37.48	36.74	NA	NA	NA	37.22
AD083	41	46.82	43.37	43.14	NA	NA	NA	43.31
AD074	43.2	47.05	42.86	42.82	NA	NA	42.82	NA
AD061	49	47.81	48.63	48.58	NA	48.63	NA	NA
AD066	50.5	47.44	47.64	47.64	NA	47.64	NA	NA
AD056	49.5	47.46	47.67	47.90	NA	47.67	NA	NA
AD007	44.5	49.00	45.89	45.80	NA	NA	45.80	NA
AD073	47	46.63	46.23	46.17	NA	46.23	NA	NA
AD079	50	48.91	47.90	48.18	47.66	NA	NA	NA
TI04	48	48.66	47.54	47.77	47.27	NA	NA	NA
AD072	43	47.35	43.69	43.60	NA	NA	NA	43.65
AD078	43.5	48.80	44.51	44.54	NA	NA	44.54	NA
AD010	57	49.66	49.58	50.23	NA	49.58	NA	NA
AD035	52	50.31	50.18	50.46	49.91	NA	NA	NA
AD008	45.5	47.42	45.58	45.59	NA	45.58	NA	NA
PD001	51.5	49.03	49.97	50.09	49.70	NA	NA	NA
AD075	44.2	47.56	47.28	47.08	NA	47.28	NA	NA
AD006	40	46.73	42.38	42.22	NA	NA	42.22	NA
AD077	47.5	47.99	47.65	47.53	NA	47.65	NA	NA
AD015	46.6	47.13	47.32	47.21	NA	47.32	NA	NA
AD014	51.3	50.07	49.73	50.04	49.62	NA	NA	NA
AD088	46	48.63	48.58	48.78	NA	48.58	NA	NA
AD129	29	38.86	34.41	33.65	NA	NA	NA	34.15
AD118	45	47.53	44.06	44.03	NA	NA	NA	44.04
AD134	44.5	47.34	44.51	44.38	NA	NA	44.38	NA
AD143	49	48.25	47.86	47.56	NA	47.86	NA	NA
AD128	39.5	45.24	40.33	40.27	NA	NA	NA	40.31
AD085	42	48.35	45.05	44.71	NA	NA	NA	44.93
AD096	42	49.95	45.87	45.66	NA	NA	45.66	NA
AD108	46.5	48.76	45.58	45.66	NA	NA	45.66	NA
AD094	44	48.63	44.50	44.45	NA	NA	44.45	NA
AD132	47.5	48.98	49.95	50.24	NA	49.95	NA	NA
AD137	48	47.40	46.70	46.35	NA	46.70	NA	NA
AD114	43.5	47.46	44.19	44.09	NA	NA	44.09	NA
AD092	37	47.04	43.10	42.98	NA	NA	NA	43.05
AD116	48.5	48.65	48.76	48.70	NA	48.76	NA	NA
AD103	50	47.90	47.62	47.86	NA	47.62	NA	NA
AD136	50	46.93	46.54	47.09	NA	46.54	NA	NA
AD099	44	47.74	44.02	44.10	NA	NA	44.10	NA
AD131	51.5	48.45	46.89	47.19	46.76	NA	NA	NA

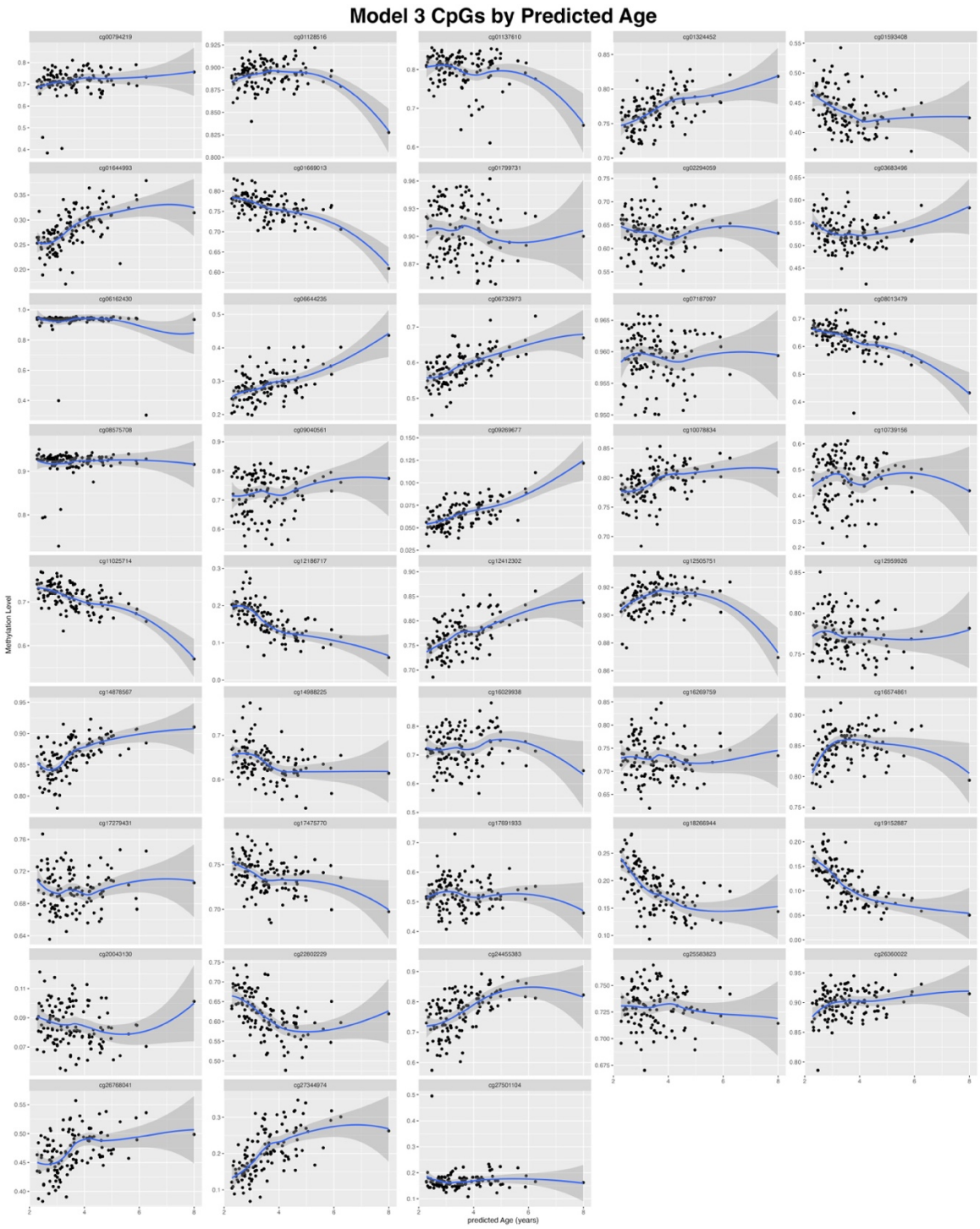
AD112	49.5	47.29	44.79	44.52	NA	44.79	NA	NA
AD151	48	48.93	50.11	49.97	NA	50.11	NA	NA
AD111	50.5	49.01	48.91	49.02	NA	48.91	NA	NA
AD086	45	48.68	47.83	47.89	NA	47.83	NA	NA
AD119	49.5	48.94	46.50	46.57	46.41	NA	NA	NA
AD135	41	46.08	42.11	41.92	NA	NA	NA	42.05
AD105	50	46.98	46.63	46.71	NA	46.63	NA	NA
AD107	43.5	48.11	44.95	44.84	NA	NA	44.84	NA
AD121	48	47.79	48.07	48.14	NA	48.07	NA	NA
AD141	48.5	47.30	45.06	45.25	NA	45.06	NA	NA
AD133	44	47.92	44.63	44.50	NA	NA	44.50	NA
AD146	53	47.78	45.82	45.84	45.83	NA	NA	NA
AD095	42	45.82	41.61	41.51	NA	NA	NA	41.58
AD125	43	47.39	43.92	43.83	NA	NA	NA	43.88
AD090	44.5	47.52	42.95	43.16	NA	NA	43.16	NA
AD150	54	49.38	49.10	49.44	48.74	NA	NA	NA
AD104	43.5	46.66	43.33	43.27	NA	NA	NA	43.32
AD139	49	48.42	47.29	47.30	47.26	NA	NA	NA
AD109	48.5	48.17	46.86	46.67	NA	46.86	NA	NA
AD113	49	48.72	47.17	47.06	NA	NA	47.06	NA
AD145	49.5	49.12	50.85	51.26	NA	50.85	NA	NA
AD106	43.5	47.86	44.21	44.15	NA	NA	44.15	NA
AD147	55.5	50.68	50.54	51.02	50.09	NA	NA	NA
AD138	49	47.91	47.87	48.09	NA	47.87	NA	NA
AD110	50	48.51	49.16	49.05	NA	49.16	NA	NA
AD153	44	48.97	45.48	45.31	NA	NA	45.31	NA
AD130	53	49.42	50.21	50.54	49.72	NA	NA	NA
AD127	53	49.63	50.36	50.66	50.03	NA	NA	NA
AD126	49	47.43	48.16	48.27	NA	48.16	NA	NA
AD117	48.5	48.63	48.08	48.25	47.80	NA	NA	NA
AD098	44	48.09	44.37	44.15	NA	NA	44.15	NA
AD115	45.5	47.91	44.60	45.00	NA	NA	45.00	NA
AD149	47.5	47.68	47.26	47.32	NA	47.26	NA	NA
AD140	43	48.15	45.71	46.00	NA	NA	46.00	NA
AD102	42	46.48	42.94	42.81	NA	NA	NA	42.90
AD089	43	47.09	42.53	42.33	NA	NA	NA	42.46
AD091	49	48.68	48.59	48.34	NA	48.59	NA	NA
AD124	49.5	45.09	44.28	43.75	NA	44.28	NA	NA
AD148	41	47.02	43.78	43.80	NA	NA	NA	43.78
AD122	48.5	47.55	47.57	47.76	NA	47.57	NA	NA

AD093	50	50.50	49.85	50.16	49.65	NA	NA	NA
AD097	42.5	46.57	43.48	43.48	NA	43.48	NA	NA
AD087	42	47.37	43.55	43.24	NA	NA	NA	43.44
AD154	46	47.41	45.67	45.61	NA	45.67	NA	NA
AD123	45	47.99	44.49	44.32	NA	NA	44.32	NA
AD144	49.5	49.46	51.00	51.22	NA	51.00	NA	NA
AD142	47	47.77	47.58	47.38	NA	47.58	NA	NA
AD120	42.5	46.75	42.82	42.83	NA	NA	NA	42.83

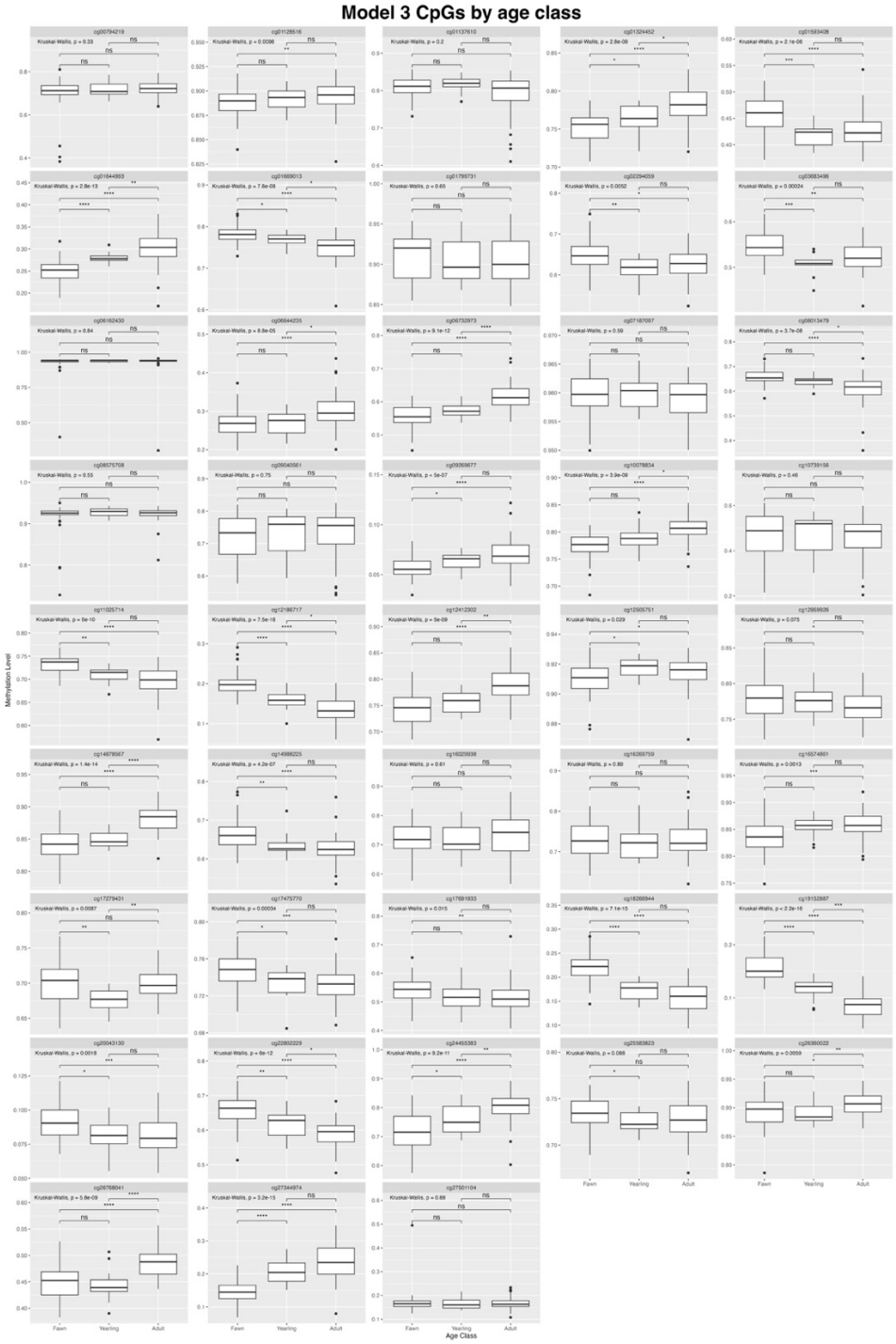
CHAPTER 3 SUPPLEMENTAL FIGURES



Supplemental Figure 1. Boxplot of methylation level across sex at all CpGs significantly predictive of hindfoot length in white-tailed deer. P-value significance level for the comparison between pairs of classes (Wilcoxon test) is represented by asterisks (*). P-value for the comparison between sexes (Kruskal-Wallis test) is reported for each CpG.



Supplemental Figure 2. Scatterplot of methylation level across predicted at all CpGs significantly predictive of hindfoot length in white-tailed deer. Regression curves are shown in blue, and standard error is represented by the shaded grey areas.



Supplemental Figure 3. Boxplot of methylation level across age classes at all CpGs significantly predictive of hindfoot length in white-tailed deer. P-value significance level for the comparison between pairs of classes (Wilcoxon test) is represented by asterisks (*). P-value for the comparison between all three classes (Kruskal-Wallis test) is reported for each CpG.