NORTHWESTERN UNIVERSITY

Tradeoffs Between Reproduction and Aging in the Human Epigenome

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

For the degree

DOCTOR OF PHILOSOPHY

Field of Anthropology

By

Calen Patrick Ryan

EVANSTON, ILLINOIS

December 2020

Abstract

Evolutionary theory predicts that reproduction entails energetic costs that detract from somatic maintenance, accelerating biological aging. In women, such 'costs of reproduction' (CoR) are thought to arise predominantly during pregnancy and lactation, while in men the physiological effects of the steroid hormone testosterone (T) are believed to be a major driver of tradeoffs in somatic maintenance. Despite support from studies in human and non-human animals, mechanisms linking CoR to somatic maintenance and aging are poorly understood. This may be due in part to a lack of a mechanistic framework than can account for the evolved, genetic underpinnings of life history traits as well as individual plasticity in life history strategies that are believed to be central to tradeoffs between reproduction and maintenance. Epigenetic processes, such as DNA methylation (DNAm), may help fill this mechanistic gap. DNAm is a chemical modification to DNA that is associated with gene activity and cellular memory and may be particularly useful for testing hypotheses about tradeoffs between reproduction and somatic maintenance. First, genome-wide DNAm can be used to gain insights into patterns of gene regulation across the genome, which can then be used to better understand untheorized biological processes that could underlie costs of reproduction. Second, DNAm can also be used to calculate epigenetic age. Epigenetic age predicts biological age and mortality, providing a measure of cellular aging and somatic maintenance. Finally, DNAm can also be used bioinformatically-impute blood leukocyte cell proportions, a proxy for immune function. In this dissertation, I leverage the power and flexibility of genome-wide DNAm from 496 young (20-22 years old) adult participants in the Cebu Longitudinal Health and Nutrition Survey to test hypotheses

about tradeoffs between reproduction and somatic maintenance. In Chapter 2, I describe epigenetic clocks in detail, including an overview of their technical application and emerging applications in human biology. I then use an epigenetic clock and telomere length – a separate measure of cellular aging – to study costs of reproduction in 394 young women in Cebu (Chapter 3). To better understand the role of the epigenome in reproduction and aging, I then examine differences in the methylome of women in differing reproductive status (Chapter 4). To aid in the interpretation of the large number of differences in DNAm between women, I then carry out a series of gene set enrichments and construct networks of biological processes. Finally, I test hypotheses about the T-associated costs of reproduction in a subset of 90 young men (Chapter 5). I examine the relationship between T and several epigenetic clocks indicative of biological aging, metabolic health, and mortality risk. To test for an immunosuppressive effect of T, I also examine bioinformatically-imputed cell type proportions, and scan the methylome for differences in T and DNAm more broadly. I show that aging based on the epigenetic clock and telomere length are both accelerated in response to gravidity, and that women appear epigenetically younger during pregnancy (Chapter 3). Differences in DNAm between nulliparous, pregnant, breastfeeding, and parous women point to changes in immune function and neurogenesis as possible biological pathways linked to aging and disease in women (Chapter 4). In men, higher T was positively related to several epigenetic clocks, although this effect was only borderline significant for two clocks, and reached statistical significance for a clock associated with metabolic health and mortality. T does not

appear to exert a clear immunosuppressive effect as indexed by our methylation-based approach, and we also did not detect differential methylation with T in this relatively small sample size (Chapter 5). In Chapter 6, I conclude by discussing the ways in which epigenetic processes are providing a mechanistic framework for studying life history traits and tradeoffs between reproduction and aging, and future directions in my research on reproduction and aging in the human epigenome.

Acknowledgements

I am eternally grateful for the many people who have trained me, supported me, challenged me, and helped me get to where I am. Doing research is only worth it if the people around you are worth it. I cannot thank you all enough for being part of this journey with me through both the struggles and the joys. Below is my incomplete and inadequate effort to put some of this into words.

First of all, I would like to thank my primary advisor, Dr. Christopher Kuzawa. Working with Chris has sharpened my writing, broadened my intellectual horizons, and taught me the high art of framing an idea to keep an audience invested and engaged. Chris has helped me understand when to push harder, when to pull back and recover, and how to see more of the big picture. I also thank Chris for bringing me into the CLHNS group and giving me the opportunity to co-write grants that will allow me to continue to play a key role in this amazing and long-standing study. Even some of our more adventuresome projects that didn't pan out (e.g. The impact of the 1918 flu on the sperm epigenome in Utah, nuclear fall-out in Norway, and Football scores in Florida) taught me to enjoy the process and take a few risks along the way. I will always think of you when I hear anything about a two-legged goat!

I thank my unofficial co-advisor Dr. Thomas McDade. His enthusiasm for bringing me in on the epigenetics work in the CLHNS helped set the stage for my dissertation, not to mention numerous very exciting and important side projects. Thom helped me see

where my work fits within broader conversations in anthropology, human biology, public health and biomedicine, and has been instrumental in bridging those gaps by introducing me to people and opportunities he thought I'd be perfect for. Bringing me to Vancouver for the CIFAR meeting in 2015 is but one example and formed the basis of numerous connections and collaborations. I could not have done it without Thom's ongoing professional and moral support. I hope we can chat over a pint at Sketchbook or Bangers and Lace in Evanston when this horrid pandemic is over.

I also thank Dr. William Leonard. Bill provided helpful feedback and comments on my dissertation. He also took the time to discuss Anthropology and Human Biology with me informally on countless occasions. Standing in the hallway or behind the annex at 1810 Hinman, Bill helped me begin to see what Biological Anthropology and Human Biology is, where it came from, why it is important, and where things might be going next. Bill's enthusiasm and kindness got me through more than one late night in the department, and his vision of the field will serve me for years to come.

My next thanks go out to Drs. Dan Eisenberg and Lee Gettler, who helped me hit the ground running by giving me the opportunity to build on my theoretical work on the human androgen receptor polyglutamine repeat, which was an absolute thrill for me at the time. Even when the research was challenging, working with them both was effortless. I especially thank Dan for handing me the reins when I really started running

with the telomere/epigenetic age paper. I'm super excited about upcoming collaborations with both of Dan and Lee. I also thank Geoff Hayes for his meetings at Fienberg early on and patience when I had the poor judgment to present him a short turnaround time ultimatum!

I thank the people at the Kobor lab who helped make my project possible, met with me, shared science and beers with me, and answered my emails or phone calls about bioinformatics and data analysis. In particular, I thank Dr. Meaghan Jones, who always shared with me her deep knowledge of epigenetics and enthusiasm for biology and has come to be a friend and colleague I look forward to working with for years to come. I also am eternally grateful to Rachel Edgar, for her generous help with bioinformatics and enrichment network pipelines. I could also not have done this work without the help others in the Kobor lab, including Dr. Michael Kobor, Dr. David Lin, Dr. Lisa McEwen, Dr. Julie McIssac, Dr. Sarah Moore, and Ms. Maggie Fu. I also thank our colleagues at University of San Carlos in the Philippines, in particular Drs. Judith Borja, Nanette Lee, and Sonny Agustin.

I am massively grateful for the time I got to spend around Drs. Alex Georgiev and Stacy Rosenbaum, both fun and brilliant scientists who enriched the group and reminded me of my training in behavioral ecology during my time at Northwestern. It's hard to describe the joy I got out of seeing Stacy starting to use R, or hearing Alex debrief about

our 2D:4D papers! I also thank Dr. Aaron Miller for always being up for a good chat, helping me think out loud and process the experience and ideas of a dissertation. Aaron and I shared more than a few eye watering laughs, which I've had a hard time finding since the pandemic and zoom became the new normal. I also thank Christina Maimone and the gang in the Northwestern R Users Group, as well as the Chicago R User Group and Chicago R ladies. I learned so much from you all, and I hope I find people as fun and eclectic to grow with in R in the future.

To my graduate student peers at Northwestern, how do I even begin to express your importance in my development as an Anthropologist, scientist, and human? I will try. During those early days, thanks especially to (in no particular order, many of them Drs.): Elisa Lenari, Ryan Lash, Kat Catlin, Rosado Ramirez, Bonnie Bright, Jared Bragg, Stephanie Levy, Paula Tallman, Morgan Hoke, Douglas Smit, Julie Perez, Kim McCabe, Tyler Barrett, Khadene Harris, V Chaudhry, Livia Garofalo, Dil Basanti, Kacey Grauer, Elizabeth Derdarian, Nazli Ozkan, Aydin Ozipek, Bilal Nasir, Jess Pouchet, Vanessa Watters Opalo and Matilda Stubbs. Later, Hafsa Oubou, Zach Nissen, Andy Kim, Elizabeth Koselka, Foroogh Farhang, Ashley Ngozi Agbasoga, and Arbeena Thapa were a source of inspiration and lessons. Still later, I had to great fortune of sharing time with İdil Özkan, Jacob Aronoff, Haley Ragsdale, Patrick Mbullo Owuor, Melissa Manus, Paula Maia, Amelia Sancilio, Stephanie Koning, Stephen Sullivan, and Bobbie Benevidez. As I write this I know I am forgetting people, but you are not forgotten! I appreciate you all. You have supported me, or pushed back at me, my ideas, or my

worldview, which might have taught me ever more. I learned a lot during my time at Northwestern, and I owe a lot of that to you all. Also, special thanks to Mallika Sarma, and my friends in the Epigenetics Journal Club, including Genevieve Housman, Chris Barrett, Mary Rodgers, Sana Saboowala, Ed Quinn, and Karthik Yarlagadda for helping me find a home at the AAPA meetings every year.

I thank my training partners and friends at Serafin Brazilian Jiujitsu. Especially thanks to Jeff Serafin, Mike Fagan, Brandon Batchelor, Jackpaul Singh, Nick Belovol, Sergei Abushevitz, Craig Fass, Rod Rodricks, Ted Lorenzen, Oleg Shilkrut, Jen Zanotti, Jacob Zweig, Ilya Bodyna, Sam Barrett, Richie Garnica and everyone else who understands the inner strength and humility that comes from surviving being strangled and broken every day. Thanks also to JT Smith at Brickhouse Gym for teaching me the fundamentals of boxing, giving me the hardest body shot I've ever had in my life, and always welcoming me to his gym with a giant smile to train when I was in Winnipeg.

I also thank my former key academic advisors on this journey. Mr. Doug Fisher for giving me my first research job as forest ecologist and teaching me about the woods, lasers, chain saws, trucks and ATVs. I thank Steve Whard, who asked me if I wanted to work in his lab during the final Cell Biology exam, and then didn't fire me when I spilled two-weeks of work by closing a eppendorf tube incorrectly. Gary Anderson, for hiring me the following summer as research assistant and 'stunt driver' to New Brunswick.

You taught me how to do research but also have fun along the way. You also hung in there with me as I struggled with the stoichiometry of elasmobranch ringer's solution – mental math still isn't a strength of mine, but computers take care of that! James Wallman at Wollongong, thanks for taking me in your lab and giving me a place to feel at home on the other side of the planet. James Hare, for teaching me the foundations of evolutionary theory - and how to come up with projects and ideas over beers. Working with you and the squirrel crew as so much fun, and earned me the nickname "Zoo Ninja". Tony Williams, for teaching me how to keep things focused, the value of null results, and giving me the independence to learn. Bernie Crespi for introducing me to Evolutionary Medicine, sharpening my ideas, and keeping me on my toes whenever I talk about evolutionary biology.

I thank my dear friends, both near and far. Ruby Fried, I miss our mental health walks along the lake, our discussions about biology and life, and our late-night chats in the lab. I could not have done it without you, and I still feel honored to call such an amazing woman my friend. Ryan Lash, you are an endless source of solace and fascination – you are a true poet and renaissance man, and I feel lucky to have friends as talented, brilliant, and genuine as you. Laura Jakul for being the friend I needed at the beginning of this journey. I thank Katie for starting this journey with me and being my anchor in the early days of my PhD. I thank Dan Simpson, Renee Lagimodiere, Pat Macklem, Neva, Keith, and Nicholas for being my unofficial extended family, inside joke refuge, and support in Winnipeg. Chris Hemsworth for sharing fires and music and friendship. I

thank Nicole for reminding me how to relax and have fun. I thank Sebastian Ibarra for being supportive, kind, honest, and hilarious and always pushing me to “look around you” and ask “why did she slap him”? Jenna, I thank for the love and support, bikerides, mini-adventures, and warm nights on the porch in Boystown. I thank Natalie Dinsdale for always being a good conversation and teaching me a little about being yourself no matter what people think. I thank Nadia Labort, Mohammed Alesa, Naif Al Ghamdi, and all the other crazy Saudis for being my family in Australia and helping me feel like a king.

I thank my mom for fighting for me in school system as a kid, asking good questions, and teaching me the art of perseverance. I thank my Dad for raising me to be skeptical and think critically, and Michele for taking care of me and showing me as a teenager how to fight for things that people are trying to keep from you. I thank Mel for teaching me about being imaginative and creative, always paving the way, and working harder than almost anyone I know. I thank Jenny for believing in me, and her family for being so welcoming and supportive. I thank Champ and Sonny for their love, lessons in patience, sacrifice, and responsibility. I thank Qadree, Troy, Jim and Sal for some of the best Hollywood beach days ever, dancing through the rain after pride, always being down for costumes, and helping me feel like family is all about loving the people around you.

Last but not least, I thank the handful of teachers that believed in me, even after being told I was a ‘problem’, didn’t fit in, needed medication, and got kicked out of multiple classes, schools, and systems. Chief among these are Monsieur Arthur Dupuis, Madame Malo, and Monsieur Philippe. I especially thank Ms. Patricia Opitz, who believed in me at just the right time, backed it up with opportunities to write and learn, and who I never got to tell what I did with that tremendous opportunity.

Most of you will never read this, I still feel like I’m missing people. If I have, I apologize. I owe everything to the people who have been on this journey with me along the way.

Table of Contents

List of Figures

List of Tables

Chapter 1. Introduction

Why do we age? And perhaps more importantly, why do some individuals appear to 'age' more quickly than others? While chronological aging can be measured by the passing of time and applies equally to living and non-living things, 'biological aging' (BA) can be seen in a progressive loss of function, reproductive viability, and increasing odds of mortality (Bulterijs, Hull, Björk, & Roy, 2015; Levine, 2013). To many, BA may seem like an inevitability. But one need only look across phylogenetic taxa to see that different species have wildly differing functional and maximum lifespans, suggestive of different rates of BA (Clark, 2002). Within animalia alone, lifespans range from less than a day for the Mayfly (*Dolania americana*), to at least 200 years for the Bowhead whale (*Balaena mysticetus*). There are even a number of species that show negligible senescence (Finch, 2009), including the Ocean Quahog clam (*Arctica islandica*), the Greenland Shark (*Somniosus microcephalus*), and even the apparently immortal Hydra (genus *Hydra*). There is also considerable variation in BA among humans, with some people living healthily beyond their first century, while others show marked declines much earlier (Bilder, 2016; Crews, 2003). Thus, although chronological age is a key predictor of mortality and functional decline in nearly every species examined, including humans, it is quite clear that chronological age and BA can be uncoupled (Levine, 2013). Nevertheless, with few exceptions, we understand very little about why species – and individuals within our own species – age at different rates, limiting our ability to predict BA in both individuals and populations as a whole. With the proportion of the world's population over 65 currently set to double in the next 30 years (World Health

Organization, 2015), understanding what causes some people to age biologically more quickly than others is both a practical and academic concern.

Biological aging and variation in BA have long been of interest to scholars and philosophers. While Plato viewed aging as a 'liberation' from the base desires and distractions of youth, Aristotle saw aging as a process of physical and moral decline (Anton, 2016). It wasn't until the 19th century that aging could be understood within an overarching theory of biological change and evolution (Darwin, 1859; Darwin & Wallace, 1858; Wallace, 1871). However, viewing aging in this framework presented naturalists of their day with a conundrum. How could Natural Selection favor the functional decline and demise of living organisms? This question would come to be taken up by August Weismann, a German evolutionary biologist whose ideas on aging and heredity are still relevant to evolutionary thinking and genetics today. He posited that because extrinsic events will come to damage individuals over time, older individuals will be less fit than younger ones. A form of 'programmed death' in cells and organisms would free up resources for younger, more fit generation, improving the fitness of the species as a whole (Weismann, 1891). This idea established the foundation for thinking about aging through an evolutionary lens, but has been criticized on the ground that it relies heavily on group selection (which fell out of favor in 20th century evolutionary thinking). More importantly, Weismann's programmed death is inconsistent with the observations that organisms can heal or even regenerate from injuries and disease, and could surely be selected to do so further.

In the 1940's, J.B.S. Haldane noted that most individuals will have already reproduced by mid-life. As a result, natural selection would invariably be weakest later in an organisms life (Haldane, 1941). In this sense, BA could be viewed not so much as an adaptive process, but as a by-product of the 'shadow' of natural selection at later ages. While there is some circularity to this argument (reproductive age and old age are defined by lifespan, and not necessarily the other way around), the accumulation of mutations that cannot be purged through negative selection came to be seen as a potential pathway through which aging and a finite lifespan could arise (Rose, Burke, Shahrestani, & Mueller, 2008). Mutation accumulation was a major advancement in evolutionary thinking about aging, but was incomplete until a key insight by the brilliant mind of George C. Williams. Apparently unaware of previous work by Haldane and Medawar, Williams pointed out that mutations causing late life declines in health and survival could not only escape negative selection, but could in fact be positively selected for if they are also associated with increased reproductive output earlier in life (Williams, 1957). This theory – referred to as antagonistic pleiotropy – was particularly important because it established a between reproduction and aging, and provided the groundwork for a subsequently influential evolutionary theory of aging: the disposable soma theory. The disposable soma theory, devised by Kirkwood (Kirkwood, 1977; Kirkwood & Holliday, 1979), is a specific framing of antagonistic pleiotropy in which it is not the genes themselves that favor early life fertility over aging and survival, but the finite nature of energy and the need to distribute it across various physiological functions. Because early life fertility is indeed selected for over late life survival as Haldane

proposed (W.D. Hamilton, 1966), processes that favor metabolic allocation to reproduction at the expense of somatic maintenance and survival are expected to evolve (S. C. Stearns, 1989). According to the disposable soma theory, it is these ‘costs of reproduction’ that are thought to accelerate BA.

The disposable soma theory falls within a broader evolutionary framework referred to as life history theory. Life history theory is based on the premise that organisms have evolved strategies that maximize their fitness under a range of contexts and conditions (Kuzawa & Bragg, 2012; Noordwijk & Jong, 1986; Partridge & Harvey, 1988). Because energy and time are finite, tradeoffs in form, function, or the timeframes within which certain behaviors can be executed create tradeoffs. Life history strategies are thought to evolve as collections of traits, because natural selection will favor the coordination of phenotypes that maximize fitness under a range of similar contexts (Réale et al., 2010). A central assumption underlying life history theory is that organisms are capable of using information from their environment and their own physiological state to calibrate strategies for growth, reproduction, storage, and bodily maintenance (Gluckman & Hanson, 2004; Gluckman et al., 2009; Kuzawa, 2005; Rando & Verstrepen, 2007; Sear, 2020; Stephen C. Stearns, 1992). Indeed, a key criticism of antagonistic pleiotropy is rooted in the fact that the same genes can be variously activated or deactivated at different points of time in the lifecourse (Mitteldorf, 2014), information lacking at the time Williams devised the theory. However, studying the kind of plasticity that is central to life history theory has been inherently difficult (Bolund, 2020). While cues with short-term relevance are integrated through rapid allostatic responses via the central nervous and

neuroendocrine systems, cues that are necessary for long-term orchestration of life history strategies – such as how much to invest in reproduction and when, how to maintain the body, and how to age – require processes that are flexible during critical windows, but robust over longer timescales (Kuzawa & Thayer, 2011; Rando & Verstrepen, 2007). Adjusting physiological set points, marking thresholds for key life history transitions, and mediating tradeoffs between competing physiological demands all necessitate a form of biological regulation and embedded 'memory' (Finch & Rose, 1995). Precisely how such sensitivity and biological memory might operate has been largely left to the imagination. More recently, however, advancements in molecular biology may be providing a way to study these questions. These advancements center around epigenetic processes.

Epigenetic processes are a collection of cellular metabolic and regulatory processes that are intricately involved in development, memory, and aging. They operate at the 'synapse' of genes and the environment, which positions them between evolved, genetic, species specific windows and the physiological and environmental cues that fine tune individual life history strategies (Boyce & Kobor, 2015; Meaney, 2010). They also have well-established roles in gene regulation and cellular memory, from early embryonic development to the maintenance of cell specific patterns at the very limits of human lifespan (Allis, Caparros, Jenuwein, & Reinberg, 2015). This creates continuity over time and provides a link between life history traits at one point in time with tradeoffs in other physiological functions at the same time or years into the future. Tradeoffs could impact health and life history traits by redirecting developmental trajectories for

individual tissues or cell lineages (Lappalainen & Greally, 2017). Because different cell lineages are associated with different epigenetic profiles, epigenetics can be used to infer cell types and hence developmental trajectories based on that uniqueness. For example, epigenetic processes can be used to algorithmically deconvolute immune cell composition in blood. This provides a way of accounting for differences in hematopoetically-derived immune profile in addition to gene regulation within the leukocytes themselves. In addition to their sensitivity to context and developmental memory, epigenetic processes are now recognized as fundamentally tied to the aging process itself. Some epigenetic changes happen stochastically, while others unfold quite predictably with time (Benayoun, Pollina, & Brunet, 2015; Jones, Goodman, & Kobor, 2015). This has allowed researchers to devise ‘clocks’ which predict chronological age with unprecedented accuracy. These clocks can also being used to study BA, providing powerful markers of mortality, health, or even other biomarkers tied to aging (Ryan, 2020).

The emerging tools built around epigenetic processes provide novel ways to study reproduction, aging, other life history traits and tradeoffs that are sensitive to context. Understanding tradeoffs around reproduction and aging may be key to understanding why we age, and why some people get sick in ways that others do not (Stephen C Stearns & Koella, 2007). But in many ways, they also challenge the paradigm of variability established over much of the 20th century. This makes it worthwhile to consider the ways that variability has been theorized in the past, and some of the ways that epigenetic processes are changing that. In this introductory chapter, I will briefly

review the history of notions about phenotypic variability and inheritance, based predominantly around genes and DNA, that were established throughout the 1900s. I will then discuss parallel research in development, ecology, and evolution, whose views of phenotypic variability and aging are often more compatible with conceptions of tradeoffs, plasticity, and life history theory described above. With this framing, I will briefly review epigenetic processes, in particular DNA methylation, and their utility for studying reproduction and aging in humans. I will then describe how I am using epigenetic methods to study tradeoffs in reproduction and aging using a long-term longitudinal cohort study in the Philippines. The overarching aim of this introduction is to provide historical and theoretical context for the chapters that follow. The goal of this research program is to use evolutionary theory and epigenetic tools to understand human evolution, health, and aging.

The Modern Synthesis and the Century of the Gene

In many ways, the 20th century was revolutionized by the gene. Beginning in 1900 with the rediscovery of Mendel's work, and ending with the first draft sequence of the human genome in 2001, our understanding of life, its origins, and its molecular underpinnings were dramatically and permanently transformed during this time. Much of this transformation can be tied to an idea that is now deeply ingrained in the consciousness of scientists and the lay person alike: the supremacy of the gene (Keller, 2000). At the time when Mendel's work was rediscovered, heredity had been a topic of contention since at least as early as the writings of Aristotle (384–322 BCE)(Villota-Salazar, Mendoza-Mendoza, & González-Prieto, 2016). Scholars could not explain how

characteristics were transmitted between generations, which is essential to understanding phenotypic variation within species as well as the diversity of life on earth. Mendel's careful observation and meticulous recordings provided robust support for a particulate theory of inheritance (Mendel, 1866), shedding light on both heredity and variation and dealing a blow to the models of blending inheritance that were prevalent at the time. As early as 1902, the 'particulate' had been narrowed down to chromosomes themselves by cytogeneticists (Boveri, 1902; Sutton, 1902). Roughly a decade later, Alfred Sturtevant (an undergraduate at the time!) demonstrated that simple traits could be accurately mapped to physical locations on chromosomes, consistent with cytogenetics and Mendel's Laws (Sturtevant, 1913). While the chemical nature of the hereditary substance was still unknown, the distinction between a so-called 'genotype' and 'phenotype' was already being made (Johannsen, 1911). Even by 1911, it would appear that the 'century of the gene', as Keller would later call it (Keller, 2000), was already underway.

The centrality of the gene to 20th century biology was further cemented with advances in theory and empirical discoveries about the biochemical structure of the genome. Fusing Mendel's particulate inheritance with Galton's work on continuous traits, Ronald Fisher, J.B.S. Haldane, and others developed mathematical and statistical theories capable of explaining how particulate inheritance could be responsible for complex, continuously-varying, traits (Fisher, 1930; R. M. Nelson, Pettersson, & Carlborg, 2013). Over time, theory also came to accommodate more complex patterns of inheritance, such as sex-linked inheritance, incomplete penetrance, co-dominance, epistasis, or pleiotropy

(Bateson, 1913; T. H. Morgan, 1910). These advances in quantitative genetics opened the door for the 'Modern Synthesis', which melded Mendel's laws of inheritance with the Darwin/Wallace theory of Natural Selection (Bowler, 2003). The modern synthesis permanently changed the face of evolutionary biology by laying the groundwork for theories about adaptation, sexual selection, aging, altruism, conflict, reciprocity, tradeoffs, and constraints (Dawkins, 2016; Fisher, 1930; William D Hamilton, 1964; William D Hamilton & Zuk, 1982; Trivers, 1972; Williams, 1966a; E. O. Wilson, 2000). Furthermore, many of the ideas of the early biometricians and evolutionary theorists extended beyond evolutionary biology, providing key tools and insights used in statistics, economics, computer science, cognitive sciences, and other disciplines.

During the first half of the 20th century empirical work in biochemistry and molecular biology also reinforced gene-centric thinking, eventually linking it with DNA specifically. Work describing the chemical structure of DNA, formalized by Watson, Crick, Franklin, Wilkins and others (Franklin & Gosling, 1953; Watson & Crick, 1953; Wilkins, Stokes, & Wilson, 2003), built on the foundations laid by Morgan and other early geneticists to describe the chemical bases by which particulate genetic information could be replicated and inherited. Equally groundbreaking was subsequent work by Crick and others deciphering the genetic code, and formulating a model of protein synthesis through RNA intermediates (Crick, Barnett, Brenner, & Watts-Tobin, 1961). These findings provided a means by which genes – physically located in DNA – could give rise to phenotypic variation, which in turn natural selection could act upon as Darwin surmised. Over the following decades, other discoveries added weight to the supremacy

of the gene, but in many ways the convergence of ideas and advancements that drove gene-centric thinking culminated in 2003 with the completion of the first draft sequence of the human genome (Lander et al., 2001). The Human Genome Project, viewed as 'the single most important project in biology and biomedical sciences' (White House Press Release, 2000), was undeniably a feat of human ambition, ingenuity, and technological innovation. It also helped establish many of the technological and conceptual foundations for contemporary genomics (Lander, 2015). But even before it was completed it was clear that the human genome project was not so much going to fulfill our expectations as transform them (Keller, 2000). Gradually, it became clear that metaphors used to market the human genome project were oversimplifications. Ideas that DNA provided the complete instructions for building a human, as reflected in ideas of it being a 'blueprint for life' or the 'genetic program', did not adequately reflect the subtle complexity of genomic processes, interactions, and the importance of the environment in human health and phenotypic variation (Eichler et al., 2010; Zuk, Hechter, Sunyaev, & Lander, 2012). The underwhelming number of uniquely human genes, the similarity of the genome to even quite distantly-related species, the amount of 'junk' DNA, and the difficulty in finding the genetic origins of many complex traits and disorders led many to view the human genome project as overhyped, oversold, under-delivering, at least in the years immediately following the HGP (Eisen, 2010; Gorski, 2010; Kamb, 2013). Adding to the confusion, molecular biologists, geneticists, and evolutionary biologists often had different conceptions about what constituted a 'gene' or 'genome' (Stencel & Crespi, 2013). These were not entirely new developments

restricted to the post-genomic era, however, and dissatisfaction with the skew towards DNA-focused, gene-centric ideas about phenotypic variability and evolution can be found throughout the 20th century (Keller, 2000; West-Eberhard, 2003).

Plasticity, differentiation, and cellular memory

The modern synthesis was based on classical genetics, in which the traits and transmission patterns of the traits themselves were the focus rather than the mechanisms and processes that give rise to them. Despite early warnings (Thomas Hunt Morgan, 1917), and evidence to the contrary, genes became synonymous with DNA. Phenotypes linked to position effect variegation (H. J. Muller, 1930), mobile transposable elements (McClintock, 1950), and imprinted genes (McGrath & Solter, 1984; Surani, Barton, & Norris, 1984) were 'exceptions to the rule' of classical mendelian inheritance and 'one-to-one' relationships between genotype and phenotype, but were often overlooked (West-Eberhard, 2003). These unusual patterns of trait development came to be understood as examples of gene regulation linked to the gene in context – either a gene in relation to the rest of the genome or the developmental milieu in which the organism took form (Keller, 2014; McClintock, 1984). The sensitivity of the genome to the environment, although differently conceived, also become central to other schools of thought in genetics, ecology, and ethology. Reaction norms – whereby the same genotype can give rise to different patterns of phenotypic expression across a range of environments – were described as early as 1909, and helped bridge the gap between quantitative genetics and phenotypic plasticity (Woltereck, 1909). The role of the environment in phenotypic variation and evolution was also central to ideas

about learning, phenotypic switching, or context-dependent life-history strategies (West-Eberhard, 2003). In a powerful response to genetic determinism and racist ideologies that are inextricably intertwined with 20th century genetics, biology, and anthropology (Marks, 2012), anthropologist Franz Boas demonstrated that rather than arising from distinct and stable racial types, traits like cranial morphology and body size among immigrants to the US were at least partly under developmental control (Boas, 1912; Boas & Boas, 1913). Thus unusual forms of heredity and plasticity sat uncomfortably alongside the DNA-focused genetics over much of the 20th century (West-Eberhard, 2003).

Other findings challenged the view that phenotypic variation belonged solely in the domain of the gene and DNA. Embryologists and developmental biologists were concerned with how a single fertilized zygote could grow to be a fully coordinated, functional multicellular organism (Villota-Salazar et al., 2016). Because geneticists had established that the DNA in each cell other than gametes was essentially identical, how cellular identity arose became central to developmental thinking. In addition to his ideas about aging and programmed death, Weismann devised a theory of development that involved the partitioning of germline and soma. The soma, he proposed, was made up of so-called *determinants* that were distributed unevenly as daughter cells branched into specialized cell lines (Weismann, 1893). This model was later falsified on the grounds that DNA itself was not partitioned but kept whole in each cell, leaving the question about what exactly led to cellular identity, and how such biological memory could remain unaltered. It was this interest in cellular identity and memory that led Conrad

Waddington to propose his famous graphical landscape in 1942, which served as a metaphor for development and cell fate (Waddington, 1942). He used the model – and the term ‘epigenesis’ – to describe the causal mechanisms linking genotype to phenotype. But by pointing to a gap between genotype and phenotype that needed to be mechanistically and conceptually filled, his insight also added a new layer to discussions about the role of genes in phenotypic variation (Gilbert, 2012). To developmental biologists following in Waddington’s footsteps it became increasingly obvious that some other process was needed for cellular differentiation and biological memory. It also became clear that genes were responsive to their environment, and phenotypes were intricately intertwined in their developmental context (McClintock, 1984). We now know that many of the key aspects of cellular identity, biological memory, and environmental sensitivity are rooted in epigenetic processes.

The 21st Century and the Rise of the Epigenome

Epigenetic processes gained their namesake from Waddington’s early ideas of cellular identity and developmental fate (Deans & Maggert, 2015; Haig, 2004; Villota-Salazar et al., 2016). They can been viewed as a collection of chemical processes and modifications that are associated with cellular differentiation and mitotically- or meiotically heritable gene expression states, in the absence of the original perturbation, and not arising as a direct result of the underlying genetic (DNA) sequence (Allis et al., 2015). Waddington’s term was popularized by Nanney (Nanney, 1958), who described it as a form of “cellular memory” and a homeostatic state based on “self-regulating metabolic patterns.” It is Nanney's definition that is closest to contemporary uses of the

term, although the term “epigenetic” is often used even in the absence of any evidence for mitotic/meiotic heritability or an effect of cell fate (Deans & Maggert, 2015; Greally, 2018; Haig, 2004).

Many epigenetic processes are intricately tied to genetic variation, thus it is true that genes are regulated by an organized program. But as researchers like John Gurdon, Kazutoshi Takahashi, Shinya Yamanaka, Keith Campbell and Ian Wilmut would demonstrate, this program is not entirely dependent on DNA sequence. Many crucial epigenetic processes linked to cellular differentiation and memory can be modified with the right combination of transcription factors. Their research in cloning and pluripotent stem cell induction demonstrated that the developmental ‘program’ can be reset or reversed, effectively rolling the developmental ball back up the slopes of Waddington’s epigenetic landscape (Campbell, McWhir, Ritchie, & Wilmut, 1996; Gurdon, 1962; Gurdon, Elsdale, & Fischberg, 1958; Takahashi et al., 2007). These findings not only revolutionize our understanding of development, but also our conceptions of memory and aging. By reprogramming a cell back its nascent state and producing an entirely new life from a fully-differentiated, aging cell, we have learned not only what makes cells remember, but how to make them forget. This work points to the idea that development, plasticity, and aging are different sides of the same coin, and that we are getting closer to bringing these facets together into a comprehensive theory of aging (Horvath & Raj, 2018). Epigenetics, therefore, is becoming synonymous with environmental sensitivity and biological memory – expanding the dominant narrative of evolution and aging built over much of the 20th century.

From a molecular perspective, epigenetic processes are currently recognized as falling into three categories: DNA methylation (DNAm), chromatin configuration involving modifications to histones, and non-coding RNAs (Allis et al., 2015). These processes are part of a set of intersecting and overlapping pathways involved in cellular metabolism, gene regulation, and genomic stability. Of these, DNAm is the most well-studied for its role in development, phenotypic variation, and disease. DNAm involves the covalent attachment of a methyl moiety to the 5' carbon of the cytosine base pair in DNA – usually in the context of a guanine to make up a 'CpG' dyad. The methyl moiety is thought to project into the major groove of DNA, inhibiting transcriptional activity (Auclair & Weber, 2012). Methylation can occur de novo through enzymatic reactions or be "copied" to the daughter strand following cell division based on the hemimethylated state of the template strand. In de novo methylation, transcription factors, enhancers, RNAs, or other co-factors are required for sequence specificity of methylation machinery. DNAm is frequently found in the context of dense clusters of CpG dyads in the promoter and regulatory regions of certain genes, referred to as CpG-islands. Highly methylated CpG islands in gene promoters are transcriptionally repressed, a state that may be passed on through mitotic, and in some cases, meiotic cell division (Ryan & Kuzawa, 2020). Accordingly, DNAm is involved in functions as diverse as embryogenesis, sexual differentiation, growth, cancer metabolism, and aging (Bocklandt et al., 2011; Fraga et al., 2005; Gupta et al., 2010; Jirtle & Tyson, 2013; Kanai & Arai, 2012; T. O. Tollefsbol, 2012).

DNA methylation and the study of life history traits and tradeoffs

DNAm offers multiple advantages for studying tradeoffs between reproduction and aging. In humans, DNAm can be measured using a methyl array, which is affordable and yields highly repeatable results across studies (Bibikova et al., 2011; Sandoval et al., 2011). Arrays like Illumina's BeadChip series (e.g. 450k, EPIC) still require technical precision and costly equipment, but are more affordable on a per sample basis than sequencing based methods, and can often be processed by in house institutional core facilities. Another major advantage of using the Illumina array technology is that there are a suite of readily available preprocessing and computational pipelines for working with DNAm data (C. Bock, 2012). This makes the analysis of DNAm using these arrays more accessible to new researchers, even those with less experience using the command line and computationally intensive methods. In some cases, DNAm data is openly-available, providing researchers interested about life history traits and tradeoffs with the opportunity to test hypotheses using pre-existing datasets before diving in on their own. This stems from another key advantage, which is that Illumina Infinium arrays form the basis for the vast majority of DNAm studies in humans, making it possible to compare results across many social and environmental contexts.

The fact that DNAm provides a window into the regulation of tens of thousands of genes, rather than a single gene or biological process, makes it a highly flexible tool for studying tradeoffs between reproduction and aging or other life history traits. Some of these changes are so predictable that researchers have devised epigenetic clocks that can be used to predict chronological age, mortality, and a broad range of biomarkers

and health outcomes (Ryan, 2020). In some cases, epigenetic clocks used to predict clinical measures and biomarkers like leptin levels or telomere leukocyte length are better of the outcomes associated with the measures than the original measure themselves (Lu, Quach, et al., 2019; Lu, Seeboth, et al., 2019), which means that DNAm from a single array provides a highly informative surrogate for numerous medically and biologically interesting processes.

Because the 450k and EPIC arrays cover some component of 99% of all refseq genes (Bibikova et al., 2011), researchers can use DNAm differences across large numbers of loci to shed light on previously untheorized biological pathways using methods such as gene ontology (Ashburner et al., 2000). When biological pathways are many, condensing findings and creating meaning through the construction of statistical and graphical networks can provide unique insights into the molecular and physiological underpinnings of life history traits and associated tradeoffs (Gillis, Mistry, & Pavlidis, 2010; Merico, Isserlin, Stueker, Emili, & Bader, 2010). This may be particularly useful in situations where conventional hypotheses have not been well-supported, creating the need to reconstruct the genomic impacts of reproduction from the molecular ‘ground up’. Leveraging databases and the huge body of literature built on studies on non-human organisms, cell cultures, genetic medicine, and genome-wide association studies make this a particularly powerful approach (Ashburner et al., 2000).

Yet another advantage of quantifying DNAm is that not only can it tell us about differences in genomic regulation between individuals, but it can also point to differences between cells within or between tissues (Houseman et al., 2012a). This is

particularly relevant because many broad physiological processes involve changes to kinds of cells in a tissue, not only the activity of the individual cells (Lappalainen & Greally, 2017). This allows for the study of differences in leukocyte composition in blood, for example, which undergird immune profile and hematopoiesis. This approach may be particularly important when studying reproduction and aging, which at the physiological level are both intricately tied to immune regulation (Finch, 2010; Fulop et al., 2018; Suchard, 2015).

New horizons in anthropological epigenetics

Until recently, most epigenetics research carried out in humans has been biomedically-focused, with a strong emphasis on the role of epigenetic processes in health-related traits like cancers, obesity, mental illness, and pre-term birth. Similarly, the majority of research on evolutionary adaptation and epigenetics has been conducted under the purview of experimental geneticists using yeast, mice, Drosophila, and Arabidopsis or other model organisms (e.g. Heo & Sung, 2011; Pillus & Rine, 1989). This work has been crucial for understanding molecular processes involved in epigenetic regulation of gene activity, and the effects of aberrant epigenetic processes, but has left open a space for understanding evolution, plasticity, and life history tradeoffs. Fortunately, the focus of epigenetic studies has started to shift. A growing number of evolutionary biologists have been working to understand adaptation and evolution in free-living organisms in the wild (e.g. Heckwolf et al., 2020; Lira-Medeiros et al., 2010), and biomedical researchers are recognizing the value of natural human epigenetic variation and including ‘diversity’ in their samples (Carja et al., 2017; Fagny et al., 2015; Gopalan

et al., 2017; Horvath et al., 2016). In anthropology, epigenetics is now being taken up enthusiastically and with exciting consequences. Anthropologists are using sophisticated theories and methods to infer phenotypes among ancient hominids (Gokhman et al., 2019, 2020), to understand human evolution in cognition or other human specific traits (Mendizabal et al., 2016; J. Zeng et al., 2012), and to build epigenetic maps of adaptation to extreme environments (Childebayeva et al., 2019). Anthropologists have also often been at the forefront of addressing the molecular and health implications of early life environments (Thomas W. McDade et al., 2017; Non et al., 2016), inequality (Kuzawa & Sweet, 2009; Thomas W. McDade et al., 2019), and trauma (Mulligan, 2016).

Despite these important contributions, one area that is surprisingly understudied in anthropological epigenetics involves questions related to life history traits and tradeoffs (Bar-Sadeh et al., 2020). Tradeoffs between reproduction and aging in women have been investigated using measures of body condition (M. Gurven et al., 2016), oxidative stress (Ziomkiewicz, Frumkin, Zhang, Sancilio, & Bribiescas, 2018; Ziomkiewicz et al., 2016), and telomere length (Barha et al., 2016; Lane-Cordova et al., 2017), but have often yielded mixed or inconclusive results. In men, the immunosuppressive effect of testosterone is thought play a key role in costs of reproduction and has also been studied with a range of immunological measures (Lee T. Gettler, McDade, Agustin, Feranil, & Kuzawa, 2014; Nowak, Pawłowski, Borkowska, Augustyniak, & Drulis-Kawa, 2018; Prall & Muehlenbein, 2015; Rantala et al., 2012). However, here too the most dominant hypotheses linking reproduction with aging have not been well-supported in

the literature and appear to fall short. What may be needed is a new way of studying tradeoffs in human life history that can both test classical hypotheses and pave the way for new ones. In the post-genomic era and in the wake of the century of the gene, one way forward may be through the incorporation of epigenetic processes like DNAm.

While biomedical studies often have disease biomarkers, and genetic and epigenetic data, they frequently lack rich, long-term information about the social and environmental context (Mill & Heijmans, 2013). In contrast, many anthropological studies have extensive details on growth, reproduction, culture, and environment, but lack large samples of individuals with genetic or epigenetic data. A rare example that combines the best of both worlds and meets the criteria outlined above for studying the tradeoffs of reproduction and aging in the epigenome can be found in the Cebu Longitudinal Health and Nutrition Survey (CLHNS)(Adair et al., 2011; Kuzawa et al., 2020). The CLHNS is a prospective community-based cohort study, drawn using a single-stage cluster-sampling procedure across 17 urban and 16 rural barangays (neighborhoods) in the Cebu metropolitan area in the Philippines (Adair et al., 2011). The study was launched by an interdisciplinary team in 1983 with the enrollment of 3327 pregnant women ranging from 14-39 years old. These women, and the children borne of those pregnancies in particular, have been followed as part of a longitudinal research covering topics as far reaching as obesity and breastfeeding to the effects of socioeconomic status, domestic violence, and substance abuse (Adair et al., 2011). In addition to this rich social and environmental information, extensive data related to life history traits and transitions has been collected on numerous occasions over the past 35 years, including

growth rate, sexual maturity and relationship status, sexual attitudes and behaviors, immune function and metabolism, parenting behavior and stress, and fertility and aging (Kuzawa et al., 2020). For the children who were in utero during study initiation (i.e. index children), these survey data are accompanied by a long list of biomarkers including hormones, cytokines, inflammatory biomarkers, and for a subset of individuals, genome-wide genetic and DNAm data.

The CLHNS provides a rare opportunity to study the epigenetics of life history tradeoffs in humans, including tradeoffs between reproduction and aging (Kuzawa et al., 2020). A number of CLHNS index children adhere to traditional Catholic ideas about sex, contraception, and marriage, and many are married young and have large families. This contributes to fecundity rates that are atypical in many Western contexts but provides a greater range of naturally-occurring variation in reproductive effort ideal for testing evolutionary hypotheses. Another advantage of the CLHNS is the narrow age-range of index children, which reduces potential for confounding brought about through secular changes to reproduction, behavior or health that could otherwise impact the epigenome. Finally, numerous waves of data collection both before and after collection of epigenetic data can help to rule out other lurking variables that might confound our findings. For example, if some unmeasured genetic, social or environmental cue increases fecundity through changes in the epigenome (and not the other way around), researchers can look at fecundity for the years following epigenetic data collection to test for this relationship. A relationship between the epigenome and fecundity is more consistent with antagonistic pleiotropy, whereas the lack of such a relationship is more consistent

with a situation in which reproduction affects the epigenome, more aligned with the disposable soma theory. Because of the lifelong data, it is also possible to account at least partly for socioeconomic status and parental education both when individuals were born and when the study was conducted. While tests like these do not eliminate the possibility for confounding, they provide ways to eliminate alternative hypotheses that are not always possible in purely cross-sectional studies.

Dissertation overview

In this dissertation, I capitalize on the lifetime of data for CLHNS index children to study tradeoffs between reproduction and aging in the human epigenome. Specifically, I concentrate on 496 young (20-22 years old) men and women with a range of lifetime exposures and reproductive histories to test the disposable soma theory. To examine the relationship between reproduction and aging in the epigenome, I use a combination of genome-wide DNA methylation data, genetic data, and reproductive histories reconstructed using multiple data collection waves in 2005 and 2007. Using multiple surveys about reproductive history allowed me to quantify pregnancies and determine women's reproductive status when DNAm was measured, even among women who were unaware of being pregnant at the time. In Chapter 2, I begin by describing 'Epigenetic Clocks', an emerging molecular tool that I use in Chapter 3 and 5 for quantifying biological aging (Ryan, 2020). I highlight the theory behind epigenetic clocks, and their utility in studying environmental sensitivity and life history tradeoffs in humans. I emphasize some of the key considerations in study design and bioinformatic processing pipelines to maximize the utility and rigor of these fascinating tools. In

Chapter 3, I use one of these epigenetic clocks (Horvath, 2013) and telomere length – a separate measure of cellular aging (Harley, Futcher, & Greider, 1990) – to test for costs of reproduction among 397 young women in the Philippines. I look at the relationship between number of pregnancies and epigenetic age and telomere length, under the premise that increased reproductive effort should come at the expense of somatic maintenance and accelerated cellular aging (Ryan et al., 2018). Because pathways contributing to tradeoffs in women may be most apparent during pregnancy and lactation, when reproductive effort is greatest, I turn to the effects of pregnancy and breastfeeding on the methylome more broadly in Chapter 4. The aim here is to understand why parity and breastfeeding are associated with differences in mortality and both increased and decreased risks for certain diseases (Beral, 1985; Grundy & Read, 2015). In order to get a better understanding of the biological processes involved in reproduction and women's health and aging, I then follow epigenome-wide association with gene set enrichment and network construction (Gillis et al., 2010; Merico et al., 2010). This provides integrated networks of biological processes that are more amenable for interpretation and hypothesis generation when looking at large numbers of epigenetic differences between women in different reproductive states. Originally, plans for the final data chapter included a second measure of DNAm and parity roughly 10 years after the original samples were collected in 2005. This would have allowed us to compare changes in DNAm with changes in reproductive effort in women, and reduce potential confounding tied to individual differences in health or access to resources. However, the global COVID pandemic interrupted the processing

of these samples, leading to a late transition to investigating costs of reproduction in men. This topic forms the basis Chapter 5, which offers a first assessment of the utility of using DNAm to study CoR in a smaller sample of men. In contrast with women, for whom reproductive investment is directly tied to pregnancy and lactation, costs of reproduction in men are thought to arise through the pleiotropic effects of testosterone (T)(Bribiescas, 2001). To test for an effect of T on mortality risk and biological aging, I examine the relationship between T and four epigenetic clocks in 90 young male index children. Each of these four clocks captures slightly different dimensions of biological aging and mortality risk, providing us a rough window into the potential costs of high testosterone in men (Hannum et al., 2013; Horvath, 2013; Levine et al., 2018; Lu, Quach, et al., 2019). To test for immunosuppressive effect of T, I examine bioinformatically-derived cell proportions, as well as immune function that has been collapsed along an axis of innate-acquired immunity. This allows us to test the classical model of costs of high T, referred to as the immunocompetence handicap hypothesis (ICHH)(Folstad & Karter, 1992; William D Hamilton & Zuk, 1982). To shed light on untheorized links between T and men's health, I then take a hypothesis generating approach to the study of the effect of T on men's epigenome (Biesecker, 2013). I conclude with future directions arising directly from this dissertation, including the current status of the longitudinal analysis of gravidity and epigenetic age in women originally planned, and other funded research to greatly expand the sample size for costs of reproduction in men. I also provide summary of some of the opportunities and

challenges that lay ahead in life history epigenetics and conclude with thoughts on the role epigenetics in studying human variation and health more broadly.

Chapter 2. ‘Epigenetic Clocks’: Theory and applications in human biology

Abstract

All humans age, but how we age – and how fast – can differ considerably from person to person. This deviation between ‘apparent’ age and chronological age is often referred to as ‘biological age’ (BA) and until quite recently tools for studying BA have remained elusive. ‘Epigenetic clocks’ have begun to change this. Epigenetic clocks use predictable changes in the epigenome, usually DNA methylation, to estimate chronological age with unprecedented accuracy. More importantly, deviations between epigenetic age and chronological age predict a broad range of health outcomes and mortality risks better than chronological age alone. Thus, epigenetic clocks appear to capture fundamental molecular processes tied to BA and can serve as powerful tools for studying health, development, and aging across the lifespan. In this Toolkit, I review epigenetic clocks, especially as they relate to key theoretical and applied issues in Human Biology. I first provide an overview of how epigenetic clocks are constructed and what we know about them. I then discuss emerging applications of particular relevance to Human Biologists – those related to reproduction, life-history, stress, and the environment. I conclude with an overview of the methods necessary for implementing epigenetic clocks, including considerations of study design, sample collection, and technical considerations for processing and interpretation of epigenetic clocks. The goals of this Toolkit are to highlight some of the ways that epigenetic clocks can inform questions in Human Biology, and vice versa, and to provide Human Biologists with the

foundational knowledge necessary to successfully incorporate epigenetic clocks into their own research.

Introduction

Aging is a ubiquitous feature of the human experience and a leading predictor of health, disability, morbidity, and mortality (Clark, 2002; Crews, 2003). Nevertheless, individuals of the same chronological age often vary markedly in their age-related physical, physiological, and cognitive decline. Rare genetic disorders such as Werner syndrome provide extreme examples of accelerated aging, but individual differences in cardiovascular, metabolic, musculoskeletal, and neurological decline are present in all populations. The decline in functional capacity that can be decoupled from chronological age is often referred to as 'biological age' (BA), and is thought to arise as a function of both genes and the environment (Levine, 2013).

Although the mechanistic underpinnings of BA are still only partly understood, the epigenome is emerging as fundamental component of BA (Benayoun et al., 2015; Jones, Goodman, et al., 2015). The contribution of epigenetic processes – including DNA methylation, chromatin modifications, and non-coding RNAs – to early development and cellular memory are well-known (Allis et al., 2015). More recently, researchers showed that the epigenome not only changes with age, but often does so in a highly predictable manner (Bocklandt et al., 2011). These predictable, age-associated changes in the epigenome have now been used to design a set of tools that are changing how we study aging across the lifespan. These tools are referred to as 'epigenetic clocks' (Field et al., 2018).

Epigenetic clocks – typically based on DNA methylation (DNAm) – were initially designed to predict chronological age, and have proven to be the most accurate tool for doing so (Horvath, 2013; Q. Zhang et al., 2019). They accurately estimate age from prior to conception to the most advanced human ages, and even 'tick' in isolated cells in vitro (Hoshino, Horvath, Sridhar, Chitsazan, & Reh, 2019). However, they also appear capable of capturing fundamental molecular processes tied to BA that are still not well understood. Epigenetic clocks outperform many other measures, including chronological age, at predicting a long list of age-related health outcomes – from cancer to menopause – as well as all-cause mortality (Horvath & Raj, 2018). Now, newer epigenetic clocks are being explicitly 'trained' not only on chronological age, but on a range of other age-related biological and health-related measures. This includes other biomarkers of health and aging such as leukocyte telomere length and leptin levels, in some cases even outperforming the predictive ability of the original biomarker of interest (Lu, Quach, et al., 2019; Lu, Seeboth, et al., 2019).

The predictive capacity of epigenetic clocks allows researchers to study aging and mortality while circumventing many of the logistical or temporal challenges inherent in following a large sample over the long timeframes necessary to obtain clinical or demographic outcomes. Newer epigenetic clocks expand their applications into new territories, solidifying them as a flexible family of tools for tackling long-standing questions in Human Biology, including plasticity in development, life-history traits, tradeoffs, and the role of the environment in the pace of aging and senescence.

The purpose of this Toolkit is to introduce Human Biologists without extensive background in genetics, molecular biology, or bioinformatics to epigenetic clocks: what they are, what they do, and how to use them. The focus of this article will be practical, and the aim is to provide readers with the foundation necessary to evaluate the utility of epigenetic clocks for their research program. After a broad introduction to the statistical methods used to 'train' epigenetic clocks – which are important for understanding the capabilities and limitations of these tools – I provide an overview of both established and emerging applications of epigenetic clocks for research in Human Biology. These include studying the impact of nutrition, exercise, and lifestyle; reproductive investment and tradeoffs; stress and resilience; and the environment and ecology on aging and age-related decline. I then discuss aspects of study design, sample collection, technical processing of DNAm, and interpretation of results for studies using epigenetic clocks. I include key references and resources that will help facilitate the incorporation of epigenetic clocks into human biology research.

What are 'epigenetic clocks'?

Epigenetic clocks can refer to two distinct but related phenomena. The first refers to epigenetic changes – typically DNA methylation (DNAm) – as they unfold predictably over time (Jones, Goodman, et al., 2015). Specifically, this definition describes an innate biological process as it relates to an age-related outcome of interest (e.g. chronological age, mortality risk, etc.). The pace or 'ticking rate' for some clocks may be influenced by genetic oscillators and circadian genes, but 'epigenetic clock' is not synonymous with other circadian 'clocks'. The second definition of 'epigenetic clock'

describes a statistical model that uses predictable epigenetic variation – often DNA methylation (DNAm) – to estimate an age-related outcome of interest (Horvath & Raj, 2018). The predictable age-related changes in DNAm that are used for epigenetic clocks can be determined using two methods: supervised machine learning or epigenome-wide association.

Most well-known epigenetic clocks are constructed using supervised machine learning methods (Horvath & Raj, 2018). This kind of clock construction entails a 'training' stage and a 'testing' stage. The clock is 'trained' by compiling a large dataset comprised of DNAm values for many samples and the outcome of interest (e.g. the chronological age of the individual the sample comes from). Sample variety and size is important for training a model that can later be generalized to other contexts (James, Witten, Hastie, & Tibshirani, 2013). Training starts with an oversaturated model in which the number of predictors (i.e. scaled DNAm for all available sites) greatly exceeds the number of observations (i.e. individual samples). Penalized regression methods (e.g. ridge, lasso, or elastic net) then either shrink (i.e. regularize) coefficients or 'soft-threshold' coefficients below an absolute value to zero. Regularizing coefficients in this way reduces the bias of the final model, making it more robust when applied in other contexts. Soft-thresholding to zero is also a form of feature selection, such that only the most informative predictors (i.e. CpG sites) are retained in the final model. The shrinkage penalty, which determines the strength of regularization and thresholding, can be 'tuned' by fitting models across a grid of penalty values and calculating the error using resampling methods such as bootstrapping or k-fold cross-validation (James et

al., 2013). The result is an equation – both a subset of CpGs and their associated coefficients – that can be then used to predict the age-related outcome of interest (e.g. chronological age) in a new sample of individuals or tissues.

Validating the model in new contexts with known outcomes is referred to as 'testing' (James et al., 2013). Testing epigenetic clocks allows the researcher to determine the accuracy of the clock for predicting the age-related outcome of interest. For example, one of the most well-studied clocks was designed by Steve Horvath (Horvath, 2013), who trained his clock using 21,369 probes from 3921 samples derived from 39 datasets comprised of 27 different tissues. His model converged on a clock that uses variation in DNAm from 353 CpG loci. This clock was then tested in 3211 samples derived from 42 different studies and from 22 tissues (Horvath, 2013). Because it was trained using data from many different tissues, Horvath's clock shows remarkable accuracy across a range of contexts and tissue types. Pearson's correlation coefficients between predicted age and chronological age are high ($r = 0.96$, $p<1.0e-200$), while median error between predicted age and chronological age are low (3.6 years across range of 0-100 years)(Horvath, 2013). Once trained and tested, the clock can be used to predict the age-related outcome of interest in new contexts.

While regularized regression and the methods described above have generated the most well-known epigenetic clocks, other approaches of selecting age-associated CpG loci have also been successfully employed. Several clocks have been derived by using the strongest correlations between DNAm and chronological age from epigenome-wide association studies (EWAS) (Lin et al., 2016; Vidal-Bralo, Lopez-Golan, & Gonzalez,

2016; Weidner et al., 2014; Y. Zhang et al., 2017). Because they may rely on relatively few CpG loci in their age calculations, such clocks can be more economical and do not require technical bioinformatic skills to use. However, it is not known whether clocks built in this way exhibit the same accuracy and robustness across contexts (e.g. across different genetic backgrounds, tissue types, age ranges) as clocks using a larger number of CpG sites. Regardless of the methods used, researchers interested in using epigenetic clocks to predict chronological age or other age-related phenomenon should understand the context in which the clock was trained and how it performs under a range of contexts relevant to the hypothesis or application. In this respect, Human Biologists also have the opportunity to play an important role in assessing performance of various clocks in different ecological and social settings as the field develops (e.g. Horvath et al., 2016).

Applications and utility of epigenetic clocks

Established applications of epigenetic clocks

Predicting chronological age

Epigenetic clocks trained on chronological age have proven to be the most accurate markers of chronological age devised (Horvath, 2013; Q. Zhang et al., 2019). This accuracy is highly desirable for forensics or for estimating chronological age in isolated populations that may not have detailed or accurate birth records. However, a ‘perfect’ chronological epigenetic clock is by design uninformative with respect to variation in health, development, and BA (Q. Zhang et al., 2019). Quite early on it became obvious

that one of the most interesting features of certain epigenetic clocks was not simply that they are accurate – but what these clocks can tell us when they are wrong! It turns out that the discrepancy between an individual's actual and predicted chronological age – the 'error' in the clock – captures a range of normal and pathological variation in biological aging and development (Chen et al., 2016; Marioni, Shah, McRae, Chen, et al., 2015). This discrepancy, often referred to as 'epigenetic age acceleration' (AgeAccel) expands the applicability of chronological epigenetic clocks beyond simple 'time-keepers' and into groundbreaking tools for studying BA.

Predicting morbidity and mortality

Human biologists and researchers in allied fields are often concerned with the long-term health of their study population, and researchers may wish to understand how healthspan and lifespan are affected by tradeoffs, constraints, lifestyle, behavior, or psychosocial stress. However, following subjects over the long periods of time necessary to record clinical and mortality outcomes can be costly to initiate and maintain (Ng et al., 2012). The ability of epigenetic clocks to predict morbidity and mortality far in advance of their clinical endpoints makes them powerful tools for studying health and BA even without long-term follow-up studies.

Horvath's clock – especially accounting for chronological age and cell-type variation in the blood sample (referred to as 'intrinsic epigenetic age acceleration' or Horvath-IEAA; Table 2-1), predicts all-cause mortality. The predictive accuracy remains even after correcting for a range of mortality-associated risk factors (i.e. chronological age, social

class, educational level, body mass index, alcohol intake, smoking pack-years, self-reported recreational physical activity, hypertension, history of diabetes, history of cancer, hypertension status, cardiovascular disease, and APOE e4 status) (Chen et al., 2016; Marioni, Shah, McRae, Chen, et al., 2015). Similarly, Hannum's clock – including correcting for chronological age and weighted to incorporate additional information about age-related blood immune cell composition ('extrinsic epigenetic age acceleration' or Hannum-EEAA) has yielded similar results (Chen et al., 2016). The relationships between epigenetic age and mortality risk reported in these studies is not trivial; individuals in the top 5% of epigenetic age were at an almost 50% higher risk of mortality during follow-up than an individual with the average epigenetic age (Chen et al., 2016).

In addition to all-cause mortality, acceleration of Horvath and Hannum clocks (and their IEAA and EEAA extensions) accurately predict leading causes of morbidity and mortality including lung function (Marioni, Shah, McRae, Ritchie, et al., 2015), frailty and cognitive decline (Breitling et al., 2016; Marioni, Shah, McRae, Ritchie, et al., 2015), and cancer (Levine, Hosgood, et al., 2015; Zheng et al., 2016). As discussed in more detail below, both clocks are also associated with a range of environmental exposures and developmental outcomes. However, neither the Horvath nor Hannum clocks were originally designed to predict these outcomes; i.e. both were 'trained' on chronological age alone. Indeed, a recently-devised epigenetic clock of chronological age using larger sample sizes is so accurate that it no longer reflects BA, and the error in this clock shows no discernable relationship with mortality (Q. Zhang et al., 2019). Accordingly,

other epigenetic clocks have been developed, trained on outcomes ranging from gestational age and leukocyte telomere length to markers of inflammation and all-cause mortality itself (Table 2-1). These new clocks are filling in the gaps left by the Horvath and Hannum clocks and are expanding the utility of epigenetic clocks into a family of molecular tools for studying aging and development across the lifespan.

While a comprehensive review of all epigenetic clocks is beyond the scope of a short review, several clocks have been devised specifically to predict BA and mortality risk, and have demonstrated exceptional capacity to do so. Levine et al. (2018) trained their 'PhenoAge (Table 2-1)' clock by selecting a subset of clinical markers predictive of age-related mortality. A mortality score (in years) was then calculated, which was used as the outcome of interest for selecting CpGs in a supervised machine learning approach similar to that described for Horvath's clock above (James et al., 2013). Thus, although it includes chronological age as a covariate, the resulting DNAm clock is based on a clinically-based mortality score, rather than chronological age itself (Levine et al., 2018). Using similar approaches, the GrimAge clock created by Lu et al. (Table 2-1) is based on chronological age, sex, and DNAm clocks for smoking pack years and 7 other clinical markers of mortality (Lu, Quach, et al., 2019). This means that GrimAge is an epigenetic clock built on sex, age, and eight other epigenetic clocks!

Although the derivation of these newer clocks may strike some readers as unintuitive, their predictive ability and accuracy across contexts is remarkable. Accelerated PhenoAge is highly predictive of cardiovascular disease risk, number of coexisting morbidities, poorer likelihood of being disease free, poorer physical and cognitive

function, and likelihood of dying of lung cancer among both smokers and non-smokers (Levine et al., 2018). In one study, the mortality hazard of individuals in the top 5% of AgeAccelPheno was found to be >2.5 times greater than those in the bottom 5% (Levine et al., 2018). Lu's GrimAge has produced even more striking results. AgeAccelGrim predicts hypertension, type II diabetes, poorer physical functioning, time-to-coronary heart disease, time-to-cancer, chronic obstructive pulmonary disease, and computed tomography for visceral adiposity and fatty liver (Hillary et al., 2020; Lu, Quach, et al., 2019). Individuals in the top 5% of AgeAccelGrim in a large meta-analysis had a mortality risk >4.5 times greater than those in the bottom 5% (Lu, Quach, et al., 2019). More recently, a clock was developed using *change* in 18 age-associated biomarkers rather than cross-sectional measurements (Belsky et al., 2020). The authors refer to this tool as referred to as a 'speedometer' of aging rather than a 'clock', stemming from the longitudinal nature of the training data and what appears to be a capacity to measure the pace – not static state – of BA. This clock or others using longitudinal data may be more useful for studying changes in environments or health interventions, such as caloric restriction (Belsky et al., 2020).

Table 2-1. List of major epigenetic clocks, how they were derived, what they were trained on, the number of CpG sites used for estimation, and relevant references.

Major Clocks	Derived from	Trained on	#CpG	References
Horvath AgeAccel	21,369 CpGs	Chronological age	352	Horvath 2013
Hannum AgeAccel	EWAS on 473,034 CpGs, feature selection on 70,387 age-associated CpG sites	Chronological age	71	Hannum et al. 2013
Horvath Intrinsic Epigenetic AgeAccel (IEAA)	Same as Horvath AgeAccel plus additional sites used for white blood cell estimation	Chronological age (estimated white blood cell proportion down-weighted)	~30k	Chen et al. 2016
Hannum Extrinsic Epigenetic AgeAccel (EEAA)	Same as Hannum AgeAccel plus additional sites used for white blood cell estimation	Chronological age (estimated white blood cell proportion up-weighted)	~30k	Chen et al. 2016
Levine-DNAmPhenoAge (AgeAccelPheno)	20,169 CpGs	Composite of clinical measures of health and lifespan	513	Levine et al. 2018
Lu-DNAmGrimAge (AgeAccelGrim)	DNAm clocks for seven candidate biomarkers and DNAm smoking pack years, age, and sex	Time to death	1030	Lu et al. 2019
Lu-DNAmTL	~450k CpGs found on both Illumina 450k and EPIC arrays	Telomere length	140	Lu et al. 2019
Weidner's 3-CpG age clock	EWAS on ~27k CpGs, feature selection on 102 CpGs	Chronological age	3	Weidner et al. 2014
Zhang's 10-CpG mortality clock	EWAS on 430,363 CpGs, feature selection on 58 replicated age-associated CpGs	All-cause mortality	10	Zhang et al. 2017

The relationships between many of the aforementioned clocks and morbidity and mortality have been replicated using large (n = 7300-9500) meta-analyses across multiple populations, even controlling for sample heterogeneity and a range of additional biological, social and environmental risk factors (but see Kresovich, Xu, et al., 2019). Furthermore, the number of sites used (71-1030 CpGs) means that these clocks appear to be relatively robust to some technical variation and/or some missing data. However, the Horvath, Hannum, Levine and Lu clocks were trained and tested on DNAm derived from the Illumina Infinium microarray platform, which may still be cost-prohibitive for some projects (see 'Choosing a clock and measuring DNAm', below). A more affordable approach includes a clock by Zhang et al. that uses a score based on DNAm at only 10 CpG sites which can be measured cheaply and easily using methods such as bisulphite pyrosequencing. Zhang's 10 CpG mortality clock (Table 2-1) is able to predict frailty, cardiovascular disease mortality, cancer mortality, and all-cause mortality with astonishing accuracy: individuals with aberrant DNAm levels for ≥5 of 10 sites have mortality hazard ratios 7 times greater than that of individuals with no evidence of aberrant DNAm for any of the 10 sites (Y. Zhang, Saum, Schöttker, Holleczek, & Brenner, 2018; Y. Zhang et al., 2017). The reliance of this clock on relatively few CpG sites for estimating morbidity and mortality risk could make this clock more sensitive to the influence of genetic or technical variation, although so far there is no evidence for the former (Y. Zhang et al., 2017). Furthermore, while Zhang's clock has not been tested in as many populations as the microarray-based clocks, these findings have been replicated in at least one other population, lending support to Zhang's 10 CpG

mortality clock for questions pertaining to mortality and morbidity in human populations (Gao et al., 2019).

Predicting growth, development, and maturation

Senescence, morbidity, and mortality represent one end of the continuum of BA. But epigenetic clocks also appear to be able to track early growth, development and maturity, making epigenetic clocks useful to researchers interested life history tradeoffs that involve growth and development (e.g. between growth and immunity (Urlacher et al., 2018)). Horvath's clock begins 'ticking' just weeks after conception, with the onset of cellular differentiation (Hoshino et al., 2019). Epigenetic aging also progresses normally in embryonic tissues grown in vitro and in pluripotent stem-cell derived organoids (Hoshino et al., 2019), consistent with the postulation that Horvath's clock captures a fundamental property of development and BA. Given that development involves the interplay of both genes and the environment, it is not surprising that the pace of epigenetic clocks appears to be determined by both genetic and early life maternal and environmental effects.

Indeed, Horvath's epigenetic clock 'ticks' most rapidly during infancy, childhood, and adolescence (Horvath, 2013). In contrast to the other clocks discussed so far, Horvath's training set included infants and children (range 0-100 years) making it more applicable to younger individuals, and most early studies of epigenetic age conducted in infants and children used this clock. Using Horvath's clock, Simpkin et al. found that infants born by cesarean section or to mothers who smoke have accelerated epigenetic age at

birth (Simpkin et al., 2016). However, some maternal effects may not manifest until childhood and adolescence. Birthweight does not predict Horvath-AgeAccel at birth, but it positively predicts Horvath-AgeAccel during childhood (~7-9 years of age) and *negatively* predicts Horvath-AgeAccel during adolescence (~14.5-19 years of age)(Simpkin et al., 2016). This 'reversal' of epigenetic age trends between childhood and adolescence is an important component of epigenetic aging: children with higher Horvath-AgeAccel may exhibit slower Horvath-AgeAccel after adolescence. It is also during childhood and adolescence when the difference in Horvath-AgeAccel between males and females emerges (Simpkin et al., 2016), with males showing markedly more advanced epigenetic ages than females by early adulthood, consistent with the higher mortality rates and shorter average life expectancy of men (Crimmins, Shim, Zhang, & Kim, 2019).

One possible explanation for the apparent reversal of Horvath-AgeAccel at puberty traces to the fact that prior to adulthood, children with favorable developmental conditions exhibit accelerated Horvath epigenetic age (Simpkin et al., 2016). Indeed, accelerated epigenetic age during childhood has been found to predict height (Lee T. Gettler et al., 2020), menarcheal age and maturational tempo (time between thelarche and menarche)(Binder et al., 2018; but see Simpkin et al., 2017). However, accelerations of Horvath's epigenetic age during childhood has also been associated with stress (Davis et al., 2017; but see Marini et al., 2020), exposure to violence (Jovanovic et al., 2017), and allergies (Peng et al., 2019), suggesting that Horvath-AgeAccel is more than simply a reflection of healthy development. Life history theory

could provide a useful framework for clarifying the divergent patterns in epigenetic aging in growing children. Children exposed to high levels of stress, violence, poor nutrition, infectious disease or extrinsic mortality may facultatively adjust maturational tempo to match presumed future prospects of survival and reproduction (Ellis & Del Giudice, 2019; Kuzawa & Bragg, 2012). Such adjustments could result in tradeoffs that will affect health and lifespan in adulthood, consistent with broader expectations of work describing the developmental origins of health and disease (DOHaD). This framework might explain the apparently paradoxical finding that children in both favorable and unfavorable environments may exhibit epigenetic age acceleration.

To more precisely model the epigenetic changes that occur during childhood, several clocks that capture gestational (Knight et al., 2016) and pediatric (McEwen et al., 2019) epigenetic age have been developed. These clocks are providing non-invasive biomarkers for understanding pre-natal developmental trajectories and will be increasingly useful for researchers working with infants and children as more data on these clocks accumulates. An important next step in this area is to link early life exposures that affect epigenetic clocks during childhood with health and functional decline during adulthood.

Epigenetic clocks as surrogates for other biomarkers

An important characteristic of epigenetic ‘clocks’ is that they can be trained on any feature that is accompanied by predictable changes in DNAm. As already described for Levine’s PhenoAge and Lu’s GrimAge clocks, this includes using DNAm as surrogate

predictors for age-related changes in other clinical or biomarkers. In addition to the GrimAge clock, Lu and colleagues generated an epigenetic clock for leucocyte telomere length, a measure genome stability and cellular senescence and biomarker for a range of health and age-related risk factors (Bakaysa et al., 2007; Blackburn & Gall, 1978; Rej et al., 2020). While the correlation between telomere length and the clock for leukocyte telomere length (DNAmTL) in validation populations was modest ($r = 0.44$), Lu et al. found that DNAmTL outperformed telomere length alone in its associations with age, sex, and self-reported racial identity (Lu, Seeboth, et al., 2019). DNAmTL also outperformed telomere length alone in predicting time-to-death, time-to-coronary heart disease, time-to-congestive heart failure, and history of smoking, even after adjusting for a range of classical risk factors (body mass index, educational history, alcohol intake, history of diabetes, history of cancer, and hypertension status)(Lu, Seeboth, et al., 2019). While DNAmTL does not appear to be capturing telomere length itself, it is thought to measure biological outcomes that are themselves associated with shortening telomere length in adults (Lu, Seeboth, et al., 2019).

The fact that the DNAmTL based surrogate clock can outperform the measure it was trained on is an unusual characteristic that has been observed for other surrogate clocks. For example, Lu's clock for smoking pack years (DNAm-PACKYRS, used for calculating GrimAge) predicts lifespan better than the self-reported smoking pack years (Lu, Quach, et al., 2019). Surprisingly, the DNAm-PACKYRS clock predicts lifespan even among non-smokers! The reasons behind this finding are unclear but have been validated in multiple independent datasets comprised of thousands of individuals (Lu,

Quach, et al., 2019). One possible explanation is that DNAm-PACKYRS is capturing some of the intrinsic biological impacts of smoking. This would make it more accurate than self-reported pack years, or better able to detect individual differences in the susceptibility to the effects of smoking itself. In most cases, the causal relationships between clinical biomarkers and their DNAm surrogate clocks remain to be elucidated. In the meantime, DNAm-based surrogate clocks for clinical biomarkers may lead to opportunities for Human Biologists. Situated at the interface of genes and the environment, DNAm surrogates may provide measures of health, development, and BA that are more proximal to the aging process itself. Furthermore, unlike some of the clinical measures they provide proxies for, DNAm can be measured in banked and/or dried blood spot samples, as will be discussed in more detail below.

Emerging applications for epigenetic clocks

Studying the impact of nutrition and lifestyle

Individual trajectories in epigenetic aging appear to take shape quite early in the lifecourse. Some of this individual variation in the ‘ticking’ of epigenetic clocks is likely set prenatally by genetic variation, which has been studied through both heritability and genome-wide association studies (Gibson et al., 2019; Lu et al., 2018; Marioni, Shah, McRae, Chen, et al., 2015). Not surprisingly therefore, longitudinal trajectories for some epigenetic clocks (i.e. GrimAge X. Li et al., 2020) are relatively stable. However, a robust measure of BA should be sensitive to many of the nutritional, behavioral, ecological, and social factors that affect the aging process (Crews, 2003). Several

epigenetic clocks appear to demonstrate such flexibility, which makes them excellent tools for studying the factors that influence BA without the need for long-term health and mortality-related outcomes.

As examples, greater self-reported consumption of fish, fruits, and vegetables are associated with lower Hannum-EEAA and AgeAccelGrim, while greater reported consumption of red meat is associated with higher AgeAccelPheno and AgeAccelGrim (Levine et al., 2018; Lu, Quach, et al., 2019). These measures of self-reported dietary intake are supported by blood measures of dietary intake; carotenoids such as lutein, lycopene, zeazanthin, α-tocopherol, and α- and β-carotenes all show negative relationships with age-corrected IEAA, EEAA, AgeAccelPheno and AgeAccelGrim (Levine et al., 2018; Lu, Quach, et al., 2019; Quach et al., 2017). Similarly, blood measures of inflammation (C-reactive protein), glucose metabolism (Insulin, glucose), systolic blood pressure, and obesity (waist-to-hip ratio, BMI) show the expected positive relationships to epigenetic aging measures, while HDL cholesterol shows similarly predicted negative relationships (Levine et al., 2018; Lu, Quach, et al., 2019; Quach et al., 2017).

Other behavioral and lifestyle characteristics that affect life expectancy are linked to epigenetic clocks. Self-reported smoking status predicts more rapid AgeAccel for mortality-trained clocks (PhenoAge and GrimAge), whereas alcohol consumption and exercise predict slower AgeAccel for these measures (Levine et al., 2018; Lu, Quach, et al., 2019; Zhao et al., 2019). There is also evidence for a beneficial effect of exercise on most of the epigenetic clocks described thus far (Levine et al., 2018; Lu, Quach, et al.,

2019; Quach et al., 2017), but the impact of physical activity may be less relevant among older individuals, perhaps because aging trajectory at that point is relatively 'set' (Sillanpää et al., 2019). Furthermore, the relationship between exercise and epigenetic age may be more complex even among young individuals. Compared to controls, young elite athletes – especially power athletes – exhibited acceleration in a less commonly used 5-CpG epigenetic clock (Spólnicka et al., 2018). Whether differences in epigenetic AgeAccel between physical training regimes (power vs. endurance) exist for more well-established epigenetic clocks (IEAA, EEAA, AgeAccelPheno, or AgeAccelGrim) – and if so, whether they are tied to the distinct metabolic or endocrinological profiles that accompany training – is an open question.

The observation that behavior and lifestyle affect the ticking of epigenetic clocks, which are themselves indicative of morbidity and mortality risk, means that epigenetic clocks may provide new tools for studying the immediate impacts of tradeoffs or health interventions. Although research in this area is still in its early phases, there is some evidence that the ticking of epigenetic clocks can be 'slowed' through changes in behavior or pharmacological means. Using a protocol designed to rejuvenate the thymus – which shrinks with age and is essential for proper immune function and cancer defense (Bilder, 2016) – Fahy et al. were able to decelerate epigenetic age acceleration for 4 clocks (Horvath-IEAA, Hannum-EEAA, AgeAccelPheno, AgeAccelGrim) by an average of 2.5 years (Fahy et al., 2019). These changes were also linked to positive indices of immune function and inflammation (e.g. C-reactive protein) and persisted at least six months after discontinuation of the protocol (Fahy et al., 2019).

A protocol involving the prolonged administration of metformin, dehydroepiandrosterone (DHEA), and recombinant human growth hormone (rhGH) like that used by Fahy et al. is unlikely to fit into research programs carried out by most human biologists. However, the treatments themselves may provide insights into broader theories about the role of hormones in mediating tradeoffs in immunity and somatic maintenance (i.e. the immunosomatic metabolic diversion hypothesis; Micheal P. Muehlenbein, 2004).The experimental use of GH or DHEA also raises questions about the extent to which natural variation in commonly studied hormones (i.e. testosterone, estrogen, cortisol, prolactin) might also affect BA as measured through epigenetic clocks (more on estrogen below). Furthermore, Fahy et al.'s intervention study raises the possibility that natural variation in social, environmental, or behavioral exposures might have similar impacts. Human biologists with expertise in biosocial and behavioral aspects of human health – from meditation and religious practices to seasonal changes in diet or exposure to sunlight – could find innovative ways to incorporate epigenetic clocks into their research.

Studying reproductive investment and tradeoffs

Few physiological transitions are as extreme as breastfeeding and pregnancy in women, which entail massive alterations in metabolism, immune function, and hormone levels (e.g. estradiol, progesterone, and human chorionic gonadotropin)(Albrecht & Pepe, 2015; Anderson, MacLean, McManaman, & Neville, 2015; Robertson, Petroff, & Hunt, 2015). The investment into reproduction – particularly in conditions of resource scarcity – are expected to lead to tradeoffs with somatic maintenance that will manifest

as accelerated BA. Evidence for a relationship between these costs of reproduction and epigenetic clocks is accumulating. While pregnant, women appear ‘younger’ than expected using Horvath’s clock, and ‘older’ than expected for both PhenoAge and GrimAge clocks (Ryan et al. unpublished; Ryan et al., 2018). Consistent with theorized ‘costs of reproduction’ that are expected to draw resources away from somatic maintenance and accelerate BA, both Horvath and GrimAge clocks also increase with gravidity (Kresovich, Harmon, et al., 2019; Ryan et al. unpublished; Ryan et al., 2018).

The effect of reproduction on epigenetic clocks may be partly tied to changes in immune cell composition of blood, but hormone levels also appear to be an important contributor to epigenetic clock ticking rate. Accelerated epigenetic age using Horvath’s clock is linked to earlier age at menopause and time since menopause in women who have already experienced this transition (regardless of the age of menopause)(Levine et al., 2016; but see Gibson et al., 2019). Furthermore, accelerated epigenetic age is observed among women who have undergone surgical menopause (bilateral oophorectomy), and appears to be decelerated with menopausal hormone therapies (Levine et al., 2016). Similarly, the severity of vasomotor symptoms (‘hot flashes’) among older women was associated with AgeAccelPheno, and women with accelerated PhenoAge and GrimAge were more likely to exhibit hot flashes (Thurston et al., 2020). These findings point to reproduction and hormones as important components in women’s epigenetic aging, and to the potential utility of epigenetic clocks for studying other key life history traits and transitions.

Less is known about epigenetic clocks and men's reproductive health and investment, although an accelerated average epigenetic age relative to women emerges shortly after puberty for several clocks (Simpkin et al., 2017), consistent with higher mortality rates among men compared to women more generally (Horvath, 2013). Given the importance of testosterone in men's life history and health (Michael P. Muehlenbein & Bribiescas, 2005), future work examining the relationship between testosterone and epigenetic clocks is warranted.

Studying psychosocial stress and resilience

Chronic stress is thought to contribute to 'wear and tear' on the body, accelerating BA (Seeman, McEwen, Rowe, & Singer, 2001). Accordingly, Hannum, PhenoAge, and GrimAge clocks are accelerated among individuals with lower household income and education levels (Levine et al., 2018; Lu, Quach, et al., 2019; Quach et al., 2017; Zhao et al., 2019). These effects may be partly tied to the effects of diet, exercise, and lifestyle described above. However, trauma and stress may also mediate the effect of these environmental factors on epigenetic age (Z. Liu et al., 2019). A significant proportion of Horvath's clock CpGs are in glucocorticoid response elements (Zannas et al., 2015). Furthermore, the administration of a synthetic glucocorticoid (dexamethosome) in living humans leads to changes in DNAm and RNA levels for genes near these CpGs (Zannas et al., 2015). Consistent with these findings, a study in adolescent girls found that greater diurnal cortisol production was linked to Horvath-AgeAccel (Davis et al., 2017). In a separate study, the number of lifetime stressors was associated with acceleration of Horvath-AgeAccel in a cohort of African American

women (Zannas et al., 2015). However, these effects were blunted among women with severe experiences of child abuse (Zannas et al., 2015), possibly mirroring blunted glucocorticoid responses often associated with such experiences (Adam, Klimes-Dougan, & Gunnar, 2007).

In fact, several other studies have found that 'resiliency' to stress might come at the cost of accelerated epigenetic age. Higher measures of self-control predict slower Horvath- and Hannum-AgeAccel, but only among individuals who were raised in the context of high socioeconomic status (SES). Individuals with higher measures of self-control raised in low SES contexts showed the opposite effect, leading the authors to conclude that resilience may only be 'skin-deep' (G. E. Miller, Yu, Chen, & Brody, 2015). Several studies among veterans also support harmful effects of resiliency on epigenetic age. Veterans exposed to traumatic events who did not show any symptoms of post-traumatic stress disorder (PTSD) were more – not less – likely to have increased Horvath-AgeAccel (Boks et al., 2015). In another study, veterans with PTSD exhibited accelerated epigenetic age compared to those without if they scored high on feelings of self-efficacy and resilience (Mehta et al., 2018). Collectively these studies suggest that stress and trauma contribute to accelerated epigenetic age, but also that those who appear to be managing it the best may experience the negative impacts on health and BA. More research is needed in this area, however, because antidepressant medications – common among individuals who score high on PTSD and major depressive disorder scales – may also accelerate Horvath's clock (Verhoeven et al., 2018).

Studying environmental and ecological variation

Given the connection between epigenetic clocks and nutrition, physical activity, reproduction, and stress, it is not surprising that epigenetic aging rate for several clocks varies across socioecological contexts (Fagny et al., 2015; McEwen, Morin, et al., 2017). In some cases, clocks display divergent age-related trends depending on the context, which may be informative about how environmental and ecological variation affects the aging process. Tsimane forager-horticulturalists of Bolivia show slower Horvath-IEAA and faster Hannum-EEAA than Caucasian or Hispanic counter-parts (Horvath et al., 2016). Slower Horvath-IEAA and faster Hannum-EEAA have also been reported for African forest-dwelling (Baka and Batwa) hunter-gatherers and forest-dwelling Agrarian Bantus, but not urban-dwelling Bantus (Gopalan et al., 2017; Horvath et al., 2016). These findings imply that accelerated immune-associated aging (Hannum-EEAA) can be accompanied by slower ‘intrinsic’ aging (Horvath-IEAA), consistent with theorized tradeoffs between immune function and development (Urlacher et al., 2018). More work is needed to establish if early life infectious environments shape the trajectory of epigenetic aging, and if so, how.

Methods and implementation of epigenetic clocks

Design and sampling considerations

Sampling, bias, and confounding

Epigenetic clocks are providing a set of highly versatile tools for studying tradeoffs in health, development, reproduction and aging. However, like any method used to study human health and variation, the robustness and generalizability of findings using epigenetic clocks hinges on addressing individual research questions while minimizing biases and confounding introduced through study design (Michels, 2012). Here I outline basic protocols for sampling that aim to avoid bias and confounding in studies of epigenetic age.

Sampling bias occurs when samples collected do not represent a random selection from the population of interest (Kahn, Kahn, & Sempos, 1989). In cohort studies, for example, attrition of individuals with higher socioeconomic status (SES) could bias the sample so that lower SES individuals are overrepresented. Because SES is in turn associated with nutrition, lifestyle, and psychosocial stress, all of which are known to alter epigenetic clocks, attrition of higher SES individuals will be reflected in changes to epigenetic age in the remaining sample. Resampling, following-up lost participants, or statistical approaches such as inverse probability weighting can minimize the impact of sampling biases.

Confounding – when an observed relationship is distorted by another, unaccounted for measure - is another challenge faced by studies of health and aging, including those using epigenetic clocks. Confounding can attenuate, amplify, falsely create, or obscure a relationship between the variables of interest and epigenetic age (Michels, 2012). For example, a study comparing the effect of physical activity on epigenetic age between urban and rural participants might be confounded by differences in diet, reproductive patterns, socioeconomic status, or exposure to pollutants. Recognizing the sensitivity of epigenetic clocks to environmental factors and including those in surveys and statistical models can help minimize the issue of confounding in studies of epigenetic age.

Epigenetic variation, including epigenetic clocks, is partly explained by genetic variation. Although most epigenetic clocks using hundreds of CpG loci appear be fairly robust to such genetic variation, cross-cultural comparison studies should consider the potential for population stratification. Including genome-wide genetic data and testing for gene x environment interactions is one way to address this issue but can be costly. Other approaches for reducing the impact of genetic variation on epigenetic age is through restricting analysis to a fairly homogeneous sector of the population, or by using statistical approaches such as mixed model association methods to model population substructure (Yang, Zaitlen, Goddard, Visscher, & Price, 2014).

Sample collection and storage

All commonly-used epigenetic clocks described here involve the quantification of DNAm. While all cells in the body contain more or less identical genomic DNA, DNAm

varies widely between tissue and cell type. For this reason, it is important to consider the tissue and cell types sampled for any epigenetic study, including epigenetic age. With the exception of Horvath's clock and McEwen's pediatric clock, most epigenetic clocks have been trained and validated using DNAm measured in blood. Even Horvath's clock, which was trained using 27 different tissues, shows some variation between tissues of the same individual. For example, obesity and Alzheimer's are most strongly linked with Horvath-AgeAccel in the liver and brain, respectively (Horvath et al., 2014; Levine, Lu, Bennett, & Horvath, 2015). In some cases, findings for one tissue type (e.g. buccal cells) may not apply to another (e.g. whole blood)(Levine et al., 2016; Slieker, Relton, Gaunt, Slagboom, & Heijmans, 2018). Unless researchers are interested in conducting their own intra-individual tissue comparisons, using the tissue on which the clock of interest has been trained or otherwise validated is the safest approach.

While most epigenetic clocks have been trained or tested using whole-blood, venipuncture can be technically demanding, often requiring a trained medical technician or phlebotomist. This can be a major limitation in many field settings, where additional challenges arise from the transportation, shipping, and storage requirements of whole blood (Thomas W. McDade, 2014). Fortunately, dried blood spots (DBS) collected on filter paper or "Guthrie cards" provide an accurate and low-cost, field-friendly alternative (Dugué et al., 2016; Ramagopalan & Rakyan, 2013). Samples are easily collected using a finger prick from small lancet and after air drying can be stored at room temperature for long periods of time. Samples stored at room temperature on Whatman FTA® cards

– which include a chemical treatment specifically developed for DNA and RNA analysis – yield stable and accurate measurements of DNAm for at least a decade (Joo et al., 2013; Walker et al., 2019). DBS therefore provide researchers with the opportunity to combine new waves of survey and data collection with banked samples from long-standing studies around the world (Ghantous, Hernandez-Vargas, & Herceg, 2018). Alternatively, researchers with ongoing studies can collect and store DBS with an eye towards including epigenetic clocks in future research.

The amount of DNA needed for epigenetic clocks depends partly on the method of measurement (array-based vs. pyrosequencing), as well as the efficiency of DNA extraction, purification, and bisulfite conversion. The bisulfite conversion step in particular can degrade genomic DNA, making methods for efficiently extracting and purifying DNA from DBS essential (Ghantous et al., 2018). For array-based methods, 250ng of bifulfite converted starting DNA is required (www.illumina.com/documentation). Sufficient quantities of bisulfite converted DNA (300-800ng) can typically be obtained from 1-2 6mm punches (600-800ng prior to bisulfite conversion). Pyrosequencing-based methods for locus-specific clocks may require slightly less starting DNA (Busato, Dejeux, Gut, & Tost, 2018).

Technical considerations

Choosing a clock and measuring DNAm

As discussed, epigenetic clocks can be characterized based on the method used to generate them, which also largely corresponds to the number of sites required for their

calculation. The most widely-used and well-validated epigenetic clocks also use the greater number of CpG sites, and were derived through the application of supervised machine learning to high-dimensional array-based datasets. These datasets come from a family of microarrays designed by Illumina (Illumina Inc., San Diego, CA). Illumina arrays are able to provide a reproducible, low bias, high density, single base resolution DNAm for hundreds of thousands of sites across the genome (Bibikova et al., 2011). Most early clocks were trained on legacy Infinium HumanMethylation27 (~27k CpG sites) and Infinium HumanMethylation450 (~485k sites) BeadChip arrays, but appear to be nearly as accurate on the newer Infinium methylationEPIC Beadchip array (~850k CpG sites) (Logue et al., 2017; Solomon et al., 2018). Other methods, such as whole-genome bisulfite sequencing, may provide alternatives for measuring clocks with many CpG sites, but are typically more expensive and often technically more challenging to work with than array-based methods. More affordable alternatives, such as microdroplet PCR (Komori et al., 2018) may eventually prove useful in the study of epigenetic age, but have yet to be validated for use with epigenetic clocks. Any variation or biases tied to the Illumina BeadChip technology that are not present when using these other methods could affect the accuracy of the clocks derived from them, making such alternatives less desirable than currently available array-based methods.

Array-based methods do have some drawbacks. Although less expensive and technically challenging to work with than next-generation sequencing, processing Illumina arrays still requires costly equipment (IScan System, Illumina Inc.), trained technicians, and strict measures of quality control. For most human biologists and

anthropological geneticists, leveraging collaborations or capitalizing on university genomics core facilities is likely the most economical and efficient way to successfully meet these demands. While the quality and cost of using core facilities can vary, most universities offer a discounted rate for larger orders or researchers affiliated with the university. At the time of writing, costs ranged from between $200-350 USD per sample, depending on the scale and researcher affiliation. Although not inexpensive, the added advantage of using array-based clocks is a wealth of additional information about the methylome. With over 850,000 CpG sites across, the Infinium methylationEPIC Beadchip array provides researchers with many opportunities to study the relationship between DNAm and health, development, and the environment beyond those offered by epigenetic clocks.

The second major method for deriving epigenetic clocks comes from epigenome-wide association studies. These clocks concentrate on predictable changes in a small number of CpG sites (i.e. < 11), making them amenable to bisulfite pyrosequencing or other targeted approaches to measuring DNAm. While bisulfite pyrosequencing is an affordable, highly-accurate method for measuring DNAm, it is not applicable to array-based clocks due to the large number of CpG sites that must be measured simultaneously – beyond a certain number of loci the cost of labor, reagents, and primers exceeds those of array-based alternatives. These targeted clocks show great promise as measures of chronological age (Garagnani et al., 2012; Slieker et al., 2018), frailty, and mortality risk (Y. Zhang et al., 2018, 2017), but have not been as well-validated as array-based clocks. As such, these clocks may provide opportunities for

researchers to run pilot studies or student projects that can later be scaled up to genome-wide, array-based methods.

When measuring DNAm using either array-based or targeted approaches, researchers should be cautious to avoid confounding the phenotype of interest with batch effects. While methods like surrogate variable analysis (Teschendorff, Zhuang, & Widschwendter, 2011) can successfully account for batch effects by themselves, analyzing samples from distinct groups, timepoints, or geographical locations together can confound technical batches with sampling procedures or the phenotypes of interest. Furthermore, batch correction during pre-processing is not recommended for some epigenetic clocks (see Box 1). To minimize technical confounding, samples that can be clearly grouped by time, exposure, geographical location or phenotype of interest should be processed and assayed in a random fashion (i.e. day, technician, chip, batch). In some cases, studies that originally utilized whole-blood for estimating epigenetic clocks may later have easier access to DNA from DBS. At other times, researchers conducting longitudinal studies may find that methods have changed since their original sample; follow-up research for samples originally run on the discontinued 450k array may need to be run on the current EPIC array, for example. Changes in either blood collection method or array platform may confound with batch effects and should be avoided if possible. When such changes are unavoidable, researchers can run a small subset of replicate samples using both methods to demonstrate the repeatability and lack of bias within samples. Although this can be costly for array-based methods, it is important for establishing validity and continuity in a study over time.

Processing DNAm and calculating array-based clocks

Compared to DNAm derived from targeted methods such as bisulfite pyrosequencing, DNAm from array-based methods requires rigorous quality control, background correction and signal normalization (Morris & Beck, 2015; Wang, Wu, & Wang, 2018). There are numerous programming tools and pipelines for carrying these procedures out, but quality control and normalization should be modified when output will be used for epigenetic clocks. It is currently possible to calculate epigenetic age for all array-based clocks (and several candidate gene clocks) using either openly-available R scripts or a single online calculator developed and maintained by Steve Horvath at UCLA (http://dnamage.genetics.ucla.edu/). More detailed instructions for the preparation and processing of raw array-based data for epigenetic clocks can be found in Box 1.

Processing steps for Illumina BeadChip array data destined for array-based clocks

- *Quality control*
    - Do not remove SNP-associated probes (Pidsley et al., 2016; E. M. Price et al., 2013)
    - Do not remove sex chromosome (XY) associated probes.
    - Do not remove poorly performing probes (<3 beads per signal or with non-significant detection p-values) from the dataset entirely.
        - One option is to create a matrix with poorly performing participant/probe cells as "NA". Immediately prior to preparing beta-values for the calculator, use this matrix to only mask those cells for which it applies with "NA". This maximizes the retention of probes that perform well for most participants and allows for Horvath's calculator to impute from the missing values from the rest.
- *Normalization*
    - Noob or quantile normalization are the recommended preprocessing methods (Fortin, Triche, & Hansen, 2017). Horvath's calculator conducts its own normalization, based on a modified version of Teschendorff's BMIQ or beta-mixture quantile normalization (Teschendorff et al., 2013).
    - Although samples should be processed to minimize confounding, batch effects should not be corrected for. Because it was trained on so many different samples from different sources, Horvath's clock is quite robust to the batch effects and correcting for them is thought to affect the normalization algorithms and epigenetic age estimates performed by the calculator. Still, researchers should avoid analyzing samples grouped by sex, age, exposure or other potentially confounding variable.
- *Cell type correction*
    - When "Advanced Analysis" is selected, Horvath's calculator estimates white blood cell counts (CD4T, CD8T, Granulocytes, Monocytes, Natural Killer cells, etc.) and these are used for some measures of age acceleration (i.e. Horvath-IEAA and Hannum-EEAA).

For array-based clocks, Horvath's website provides a detailed tutorial on additional formatting for the calculator and interpretation of the results. Briefly, there is a 'basic analysis' in which users simply provide a .csv file containing beta-values for roughly 30k pre-selected CpG loci for each participant (columns). This will provide an estimate of BA

using Horvath's clock and a standard quality statistic for each participant. There is also an 'advanced analysis for blood', which requires an additional .csv file containing columns containing participant id, age, and sex. The additional file quality control checks for consistency between predicted and reported tissue type and sex (based on X chromosome DNAm). More importantly, the advanced analysis option automatically calculates clocks that include chronological age in their estimates (Horvath-IEAA, Hannum-EEAA, PhenoAgeAccel, GrimAgeAccel, etc.) as well as surrogate clocks for a number of blood biomarkers (e.g. Telomere length, Leptin, PAI1, Cystatin), and estimates of white blood cell counts (eg. CD4T, CD8T, B cells, Natural Killer cells, Monocytes etc.). The range of outputs lend Horvath's calculator great flexibility for a variety of research questions relevant to human biologists.

As discussed, most epigenetic clocks are trained directly on chronological age or measures closely tied to age-related decline. However, most researchers are interested in acceleration of BA compared to others of the same age: does this person look biological 'older' or 'younger' than we expect, and why? In other words, the raw output of an epigenetic clock is sometimes not the variable of interest. Estimates for age acceleration can be derived from the difference between epigenetic age and chronological age (epigenetic age – chronological age) or the residuals of a regression of epigenetic age on chronological age (epigenetic age ~ a + b*chronological age, where a is the intercept and b is the slope). Alternatively, researchers can include chronological age with other variables in the model and epigenetic age as the outcome. As previously discussed, systematic differences between predicted and chronological

age may arise from DNA degradation during storage or processing, making it important to randomize across chips and to maintain as close a sampling protocol as possible if comparing different populations.

For candidate gene clocks, genomic loci, coefficients, as well as primers are available in the results or supplementary sections of the relevant sources (Weidner et al., 2014; Y. Zhang et al., 2017). Weidner's age estimate can also be predicted using a free online calculator (http://www.molcell.rwth-aachen.de/epigenetic-aging-signature/). Additional candidate gene clocks for chronological age, mortality, or other outcomes that are of interest to human biologists will no doubt continue to be developed.

Conclusions

Epigenetic clocks are groundbreaking tools that are changing how researchers study human development, aging, and health. They are providing insights into fundamental molecular processes underpinning health and aging and are emerging as important biomarkers of those processes. But the pace of 'aging' and how that plays out for any individual person is a complex interaction between evolutionary, social, and cultural forces. As a discipline, Human Biology strives to weave together these aspects of the human condition, and can both benefit from and contribute to our understanding of the epigenetics of aging and epigenetic clocks.

Chapter 3. Reproduction predicts shorter telomeres and epigenetic age acceleration among young adult women

Abstract

Evolutionary theory predicts that reproduction entails costs that detract from somatic maintenance, accelerating biological aging. Despite support from studies in human and non-human animals, mechanisms linking 'costs of reproduction' (CoR) to aging are poorly understood. Human pregnancy is characterized by major alterations in metabolic regulation, oxidative stress, and immune cell proliferation. We hypothesized that these adaptations could accelerate blood-derived cellular aging. To test this hypothesis, we examined gravidity in relation to telomere length (TL, n=821) and DNA-methylation age (DNAmAge, n=397) in a cohort of young (20-22 year-old) Filipino women. Age-corrected TL and accelerated DNAmAge both predict age-related morbidity and mortality, and provide markers of mitotic and non-mitotic cellular aging, respectively. Consistent with theoretical predictions, TL decreased (p=0.031) and DNAmAge increased (p=0.007) with gravidity, a relationship that was not contingent upon resource availability. Neither biomarker was associated with subsequent fertility (both p>0.3), broadly consistent with a causal effect of gravidity on cellular aging. Our findings provide evidence that reproduction in women carries costs in the form of accelerated aging through two independent cellular pathways.

Introduction

Evolutionary theory predicts that energy expenditure in the form of reproductive effort comes at the expense of somatic maintenance and lifespan (Stephen C. Stearns, 1992). Because resources are finite and selection favors early life fecundity over late life functional decline (Williams, 1957), reductions in somatic maintenance driven by the 'costs of reproduction' (CoR) are expected to accelerate senescence and functional decline and increase mortality risk (Harshman & Zera, 2007; Kirkwood, 1977). When extrinsic mortality is high or resources are limited or unpredictable, selection will favor future discounting and a shift towards 'faster' life-history strategies (Nettle, 2010; Stephen C. Stearns, 1992). While potentially adaptive from an evolutionary point-of-view, investing less into growth and maintenance and more into reproduction early in life could compound tradeoffs between reproduction and longevity and thereby accelerate senescence (Jasienska, Bribiescas, Furberg, Helle, & Núñez-de la Mora, 2017; Kuzawa, 2007; Williams, 1957).

CoR have been demonstrated in animal models, whereby reproduction hastens senescence (Maynard Smith, 1958; D. Reznick, 1985); conversely, selection for late life fecundity results in lifespan extension (Curtsinger et al., 1995; Rose et al., 2002). In humans, CoR has been predominantly studied through the use of historical datasets, which show that increased reproductive effort is often associated with a shortening of lifespan (Westendorp & Kirkwood, 1998; Doblhammer & Oeppen, 2003; Penn & Smith, 2007; Gagnon et al., 2009; Bolund, Lummaa, Smith, Hanson, & Maklakov, 2016; but see Le Bourg, 2007), and that these costs are exacerbated when resources are limited

(Dribe, 2004; Lycett, Dunbar, & Voland, 2000; Tracer, 1991). However, most studies of CoR in humans are restricted to modeling mortality as the sole outcome, and are therefore unable to address the underlying biological processes through which CoR might translate into senescence and functional decline.

Among women, CoR likely accumulate predominantly during lactation and pregnancy (Jasienska, 2009; J. Speakman & Król, 2005). Lactation is energetically taxing, while the highly invasive hemochorial placentation of human pregnancy places substantial physiological and immunological demands on the female body (Peter Thorpe Ellison, 2009; Emery Thompson, 2013; Soma-Pillay et al., 2016). At the cellular level, pregnancy-induced senescence may be mediated through mitotic or non-mitotic pathways, or both. Mitotic – or replicative – cellular aging can be measured using telomere length (TL). Telomeres are non-coding DNA sequences that cap chromosomes, and are required for cell division and survival (Blackburn & Gall, 1978; Meyne, Ratliff, & Moyzis, 1989). Telomere length shortens with cell division and chronological age, placing a limit on the number of cell divisions (Harley et al., 1990; Olovnikov, 1971; Richter & Zglinicki, 2007). At a critical threshold, TL attrition leads to the exhaustion of a cell's proliferative potential, a process referred to as 'cellular senescence' (Fulop et al., 2018; Sidler, Kovalchuk, & Kovalchuk, 2017). Shorter TL controlling for age in turn predicts higher morbidity and mortality rates (Bakaysa et al., 2007; Cawthon, Smith, O'Brien, Sivatchenko, & Kerber, 2003; Haycock et al., 2014; Kimura et al., 2008).

Pregnancy may also affect cellular aging through pathways operating independently from TL (Lowe, Horvath, & Raj, 2016). A powerful emerging marker of non-mitotic cellular aging is epigenetic age (DNAmAge)(Lowe et al., 2016). DNAmAge in human (Horvath, 2013) and non-human genomes (Stubbs et al., 2017; Thompson, vonHoldt, Horvath, & Pellegrini, 2017) is calculated from methylation at a species-specific subset of cytosine-guanine dyads (CpGs), and is strongly correlated with chronological age (Jones, Goodman, et al., 2015). Independent of a host of associated risk factors in humans, accelerated DNAmAge relative to chronological age is associated with elevated risks for morbidity and mortality (Chen et al., 2016; Christiansen et al., 2016; Marioni, Shah, McRae, Chen, et al., 2015). Vital to capitalizing on epigenetic age as a marker of non-mitotic cellular aging, accelerated DNAmAge predicts senescence and mortality independently of TL in living humans (Breitling et al., 2016; Marioni et al., 2016), and independently of both TL and the DNA damage response in vitro (Horvath, 2013; Lowe et al., 2016).

Human pregnancy could generate costs to female health and lifespan by shortening TL (mitotic age), accelerating DNAmAge (non-mitotic age), or both. During pregnancy, blood cells proliferate to compensate for fluid volume expansion (Bauer, 2014; Lurie, Rahamim, Piper, Golan, & Sadan, 2008), and women experience a shift towards innate immunity and an increased sensitivity to infection (R. H. Gray et al., 2005; Kraus et al., 2012; Lanciers, Despinasse, Mehta, & Blecker, 1999; Roberts, Satoskar, & Alexander, 1996). Data from cell culture, rodent based experiments, and clinical studies show that inflammation and infection increase cell proliferation and DNA damage, both expected

to accelerate the pace of telomere shortening (Aviv et al., 2006; Carrero et al., 2008; Farzaneh-Far et al., 2010; O'Donovan et al., 2011; Pommier et al., 1997; Sampson, Winterbone, Hughes, Dozio, & Hughes, 2006; Sanders et al., 2012; Solorio et al., 2011). Accelerated DNAmAge relative to chronological age has been observed in other pro-inflammatory contexts (Horvath & Levine, 2015; Kananen et al., 2015), and with menopause (Levine et al., 2016), an important physiological and life history transition in human females. DNAmAge acceleration arising from menopause, whether naturally-occurring or surgically-induced, was attenuated by hormone therapy (Levine et al., 2016), suggesting that physiological and hormonal changes like those accompanying pregnancy could have effects on DNAmAge. While recent studies examining TL or DNA damage and pregnancy have yielded mixed results (Barha et al., 2016; Lane-Cordova et al., 2017; Ziomkiewicz et al., 2018, 2016), none have attempted to test for CoR in humans using mitotic and non-mitotic measures of cellular aging simultaneously.

Here, we test for human CoR using mitotic (TL) and non-mitotic (Horvath's DNAmAge (Horvath, 2013)) measures of cellular aging. We test three inter-related hypotheses in a relatively young cohort (age 20-22) of women in the Philippines. First, we ask whether pregnancy history increases mitotic or non-mitotic measures of cellular aging, or both (H1). We consider whether any associations between reproductive history and cell aging are stronger among women of lower socioeconomic status, for whom resource constraints are expected to be highest (H2). Finally, we evaluate the potential for reverse causation by examining the effect of both TL and DNAmAge on the number of pregnancies over the subsequent 4 years (H3).

Methods and Materials

Data collection

Data came from the Cebu Longitudinal Health and Nutrition Survey (CLHNS), a birth cohort study in Metropolitan Cebu, Philippines that began with enrollment of 3,327 pregnant mothers in 1983-1984 (Adair et al., 2011). Longitudinal data are available for download at: https://dataverse.unc.edu/dataverse/cebu. In 2005 blood samples from overnight fasted subjects were collected into EDTA-coated vacutainer tubes. Automated and manual DNA extraction (Puregene, Gentra) was conducted on blood samples. Informed consent was obtained from all participants and data collection was conducted with approval and in accordance with the Institutional Review Boards of the University of North Carolina at Chapel Hill and Northwestern University.

Telomere length

TLs were measured using a modified form of the monochrome multiplex quantitative polymerase chain reaction assay that was externally validated. Details of the protocol and external validity can be found in (D. T. Eisenberg, Kuzawa, & Hayes, 2015) and since the coefficient of variation has recently been recognized to be an invalid statistic to assess TL measurement reliability (D. T. Eisenberg, 2016; Verhulst et al., 2015), intraclass correlation coefficient statistics of measurement error can be found in (D. T. A. Eisenberg, Borja, Hayes, & Kuzawa, 2017).

Epigenetic age (DNAmAge)

160ng of sodium bisulfite converted DNA (Zymo AZDNA methylation kit, Zymo Research, Irvine, CA, USA) was applied to the Illumina HumanMethylation450 Bead Chip using manufacturer's standard conditions. Standard methods for background subtraction and color correction were carried out using default parameters in Illumina Genome Studio and exported into R for further analyses. Quality control involved first confirming participant sex and replicate status. This was followed by quantile normalization using *lumi* (Du, Kibbe, & Lin, 2008) on all probes including SNP-associated and XY multiple binding probes. To maximize the number of sites available for the epigenetic age calculator, probes with detection p-values above 0.01 were called NA for poor performing samples only, and were otherwise retained. Horvath's DNAmAge was calculated using an online calculator (http://labs.genetics.ucla.edu/horvath/dnamage/), designed to be generally robust to cell-type differences associated with age (Horvath, 2013). Background-corrected beta values were pre-processed using the calculator's internal normalization algorithms.

Socioeconomic status (SES)

SES is measured as a combination of income, education, and assets. Participants reported their annual income from all sources, including in-kind services, and the sale of livestock or other products by household members during the prior year, which were summed to determine total household income. Incomes were deflated to 1983 levels, and log-transformed. Maternal education (in years) was also reported. Participants also

reported on nine assets (coded 0, 1) that were selected to capture population-relevant aspects of social class, including electricity, televisions, refrigerators, air conditioners, tape recorder, electric fans, jeepneys, cars, and their residence. In addition, house construction type (i.e., light, mixed, permanent structure) was coded as 0,1, and 2, respectively. Thus, asset scores ranged from 0 to 11. A principal components analysis was run on log income and assets at birth (1983) and at sample collection (2005) along with maternal education in Stata (v. 14.1). The first component of variation accounted for 49% of the variation and individual scores for this component of variation were used as our measure of SES.

Statistical methods

The key predictor variable was gravidity (the number of pregnancies including stillbirths, miscarriages and live births, but not current pregnancies) the respondent reported having had in 2005 (at the time of blood sampling). Control variables included chronological age in 2005 (the time of blood collection), the measure of socioeconomic status (SES) described above, average urbanicity score between 1983 and 2005 (Dahly & Adair, 2007), and whether the respondent was pregnant at the time of blood collection. Pregnancy status was reported at the time of sampling, and through back-calculation based on parturition within 9 months of the original interview (maternal and infant measures are recorded with each pregnancy as part of ongoing tracking process). DNAmAge acceleration refers to DNAmAge residualized on chronological age. Principal components (PCs) of genome-wide genetic variation were included to control for potential population genetic structure. The derivation of these principal components has

been described previously (Croteau-Chonka et al., 2011, 2012; Wu et al., 2012). As in previous analyses (Bethancourt et al., 2015; D. T. A. Eisenberg et al., 2017), the bivariate association between the first ten principal components and TL were tested. The top principal components up to and including the last one showing a significant bivariate association with TL (10 total) were retained as control variables, with the same 10 principal components used for DNAmAge models.

Linear regression was used for analyses predicting TL and DNAmAge (both normally distributed continuous outcome variables), while generalized linear models with a Poisson family and log-link were used to test for reverse association – that TL/DNAmAge would predict gravidity (a discrete integer) over the subsequent 4 years. The negative effect of time between 2005-2009 surveys and number of pregnancies during this time is an artifact tied to household visit schedules and urbanicity (less urban participants tend to have more pregnancies, and were visited later in the data collection wave). All models were two-tailed with $\alpha = 0.05$ and were followed by standard model diagnostics (J. Fox & Weisberg, 2011). For all linear regressions, the absence of collinearity in predictor variables was confirmed with variance inflation factors (VIFs) for all models falling below 1.1, while Poisson GLMs showed no signs of under- or over-dispersion (Kleiber & Zeileis, 2008). Despite the large number of nulliparous women and relatively small number of women with 3 or more pregnancies, all model assumptions were met, and there was no evidence of heteroscedasticity, outliers, or high leverage data points confounding our analyses. All analyses were run in R (R Core Development

Team, 2016) with ggplot2 (Wickham & Chang, 2013) and stargazer (Hlavac, 2014) for figures and tables.

Results

The relatively young women in our sample (21.7 ± 0.4 years old) displayed a range of reproductive histories. While women who had never been pregnant formed the largest group (n=507; 61.7%), women having experienced one (n=174; 21.2%), two (n=102; 12.4%), and three (n=28; 3.4%) pregnancies were also well represented. A small subset of women had experienced four (n=7; 0.8%) or five (n=3; 0.4%) pregnancies. Although the women in our sample fell into a relatively narrow age range, age-adjusted measures of DNAmAge and TL were themselves uncorrelated (p=0.64; n=396), consistent with their independent roles in cellular aging.

TL decreased and DNAmAge acceleration increased with the number of pregnancies in a woman's reproductive history (Fig. 3-1; Table 3-1). The relationship between gravidity and both measures were also relatively robust – in nested models controlling for a range of potential confounders, effects sizes for pregnancy number remained stable or increased (Table 3-1). Each additional pregnancy was associated with the equivalent of 0.34-3.67 years of telomere aging, and 0.29-0.63 years of DNAmAge acceleration.

Table 3-1. Regression models linking number of pregnancies to telomere length (models 1–4) and DNAmAge (models 5–8). Models marked † include controls for top 10 principal components of genetic variation and average urbanicity score. *Note:* +$p < 0.1$; *$p < 0.05$; **$p < 0.01$.

	Telomere Length				**DNAmAge**			
	(1)	**(2)†**	**(3)†**	**(4)†**	**(5)**	**(6)†**	**(7)†**	**(8)†**
Age	−0.047	−0.029	−0.028	−0.029	0.485	0.667	0.656	0.645
p-values	0.003**	0.071+	0.073+	0.068+	0.293	0.157	0.158	0.165
No.Pregnancies	−0.014	−0.013	−0.014	−0.016	0.363	0.326	0.459	0.510
p-values	0.025*	0.039*	0.031*	0.020*	0.026*	0.049*	0.007**	0.005**
SES		−0.006	−0.006	−0.004		−0.180	−0.214	−0.291
p-values		0.143	0.161	0.395		0.146	0.081+	0.055+
Currently Pregnant (Y)			0.011	0.011			−1.472	−1.460
p-values			0.534	0.540			0.001**	0.001**
No. Pregnancies × SES				−0.004				0.106
p-values				0.362				0.385
Intercept	1.826	1.337	1.332	1.343	14.818	10.319	10.611	10.850
Observations	821	821	821	821	397	397	397	397
Adjusted R^2	0.015	0.063	0.062	0.062	0.011	0.041	0.067	0.067

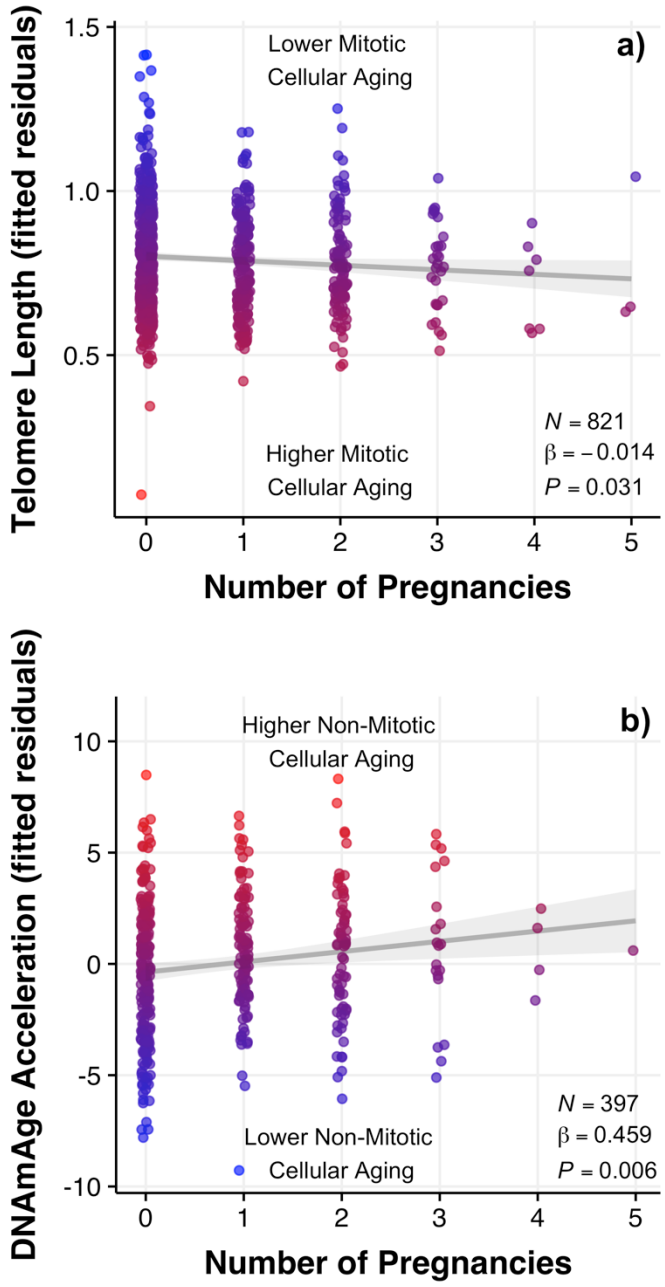

Figure 3-1. Relationship between mitotic (TL) and non-mitotic (DNAmAge acceleration) measures of cellular aging and reproductive history (number of pregnancies) in young women. (**a**) Residualized TL for all variables in Table 2, Model 3, and statistics from same model. (**b**) Residualized DNAmAge for all variables in Table 2, Model 7, and statistics from same model. Graphs are labeled and dots are colored by relative aging for each marker (blue, low; red, high) and best fit lines are drawn with 95% CI of beta value.

In contrast with the apparent effect of gravidity, being currently pregnant was associated with a decrease in DNAmAge (Table 3-1). Current pregnancy status obscured the relationship between gravidity and DNAmAge, evident from the increase in the regression coefficient linking gravidity with DNAmAge with its inclusion (Table 3-1, models 7 and 8 vs. 5 and 6). Contrary to our prediction, the relationship between gravidity and our measures of cellular aging was not affected by socioeconomic status, a proxy for resource availability in this population (Table 3-1).

We also tested for reverse causation by examining the associations of TL and DNAmAge with future reproduction. Neither measure of cellular aging at the time of measurement (2005) predicted the number of pregnancies over the subsequent four years (2005-2009), whether or not we controlled for baseline gravidity in 2005 (Table 3-2).

Table 3-2. Relationship between telomere length (TL) and epigenetic age (DNAmAge acceleration) measured in 2005 and parity over the subsequent four years (2005–2009). Models with and without adjustment for baseline gravidity in 2005.

	Parity 2005-2009			
	Age Adjusted TL~		**Age Adjusted DNAmAge~**	
	(1)	(2)	(3)	(4)
Measurement time bt. 2005-2009 (Days)	-0.003	-0.003	-0.002	-0.002
	p = 0.004**	p = 0.009**	p = 0.058+	p = 0.068+
Parity in 2005		0.252		0.123
		p = 0.000**		p = 0.016*
Age Adjusted Telomere Length in 2005	0.059	0.148		
	p = 0.835	p = 0.601		
Age Adjusted DNAmAge in 2005			-0.011	-0.016
			p = 0.483	p = 0.325
Intercept	4.457	3.777	3.460	3.265
	p = 0.006**	p = 0.022*	p = 0.062+	p = 0.082+
Observations	743	743	397	397
Log Likelihood	-836.740	-818.205	-485.276	-482.433
Akaike Inf. Crit.	1,679.481	1,644.411	976.552	972.866

Note: +p<0.1;*p<0.05;**p<0.01;***p<0.001

Discussion

TL and DNAmAge, measures of mitotic and non-mitotic cellular aging, respectively, were both associated with reproductive history in our sample of young women. The relationship between gravidity and cellular aging was relatively robust to a number of potential confounders, and did not appear to be mediated by socioeconomic status, a measure of resource availability. Moreover, neither measure was associated with gravidity over the subsequent 4 years, consistent with a causal effect of the number of pregnancy on cellular aging.

Although consistent with theoretical predictions and non-human animal work, this is the first study to our knowledge to examine CoR using both mitotic and non-mitotic measures of cellular aging. Gravidity predicted age-related changes in both TL and DNAmAge in our study, yet several recent studies of CoR in women using TL alone did not find the predicted relationship. The first, conducted among 75 Guatamalan Maya women, reported a positive association between TL and number of surviving offspring over a 13-year period (Barha et al., 2016). TL in that study was determined using a combination of saliva- and buccal-derived DNA samples, which unfortunately have not been consistently associated with chronological age (Goldman et al., 2017; O'Callaghan et al., 2008; Thomas, 2008). Furthermore, two separate measures of TL in that study were uncorrelated within individuals between the two timepoints, making comparisons between these findings and our own blood-derived TL findings difficult.

Contrasting with our findings, a study among 620 participants of the US-based CARDIA study did not find evidence for any relationship between parity and TL (Lane-Cordova et al., 2017). Why this study found no evidence for an effect of parity on TL while our findings support CoR is unclear, but could relate to pronounced differences in the age ranges and socio-ecological conditions in the two populations. Notably, markers of oxidative stress appear to be affected by parity in some socio-ecological contexts but not others (Ziomkiewicz et al., 2018, 2016). Furthermore, TL attrition occurs more rapidly at younger ages (Frenck, Blackburn, & Shannon, 1998), suggesting that any impacts of reproduction on TL shortening could be most pronounced among young women, especially if reproduction begins in adolescence and overlaps with late stages

of the mother's own somatic growth (K. Hill & Kaplan, 1999; Stephen C. Stearns, 1992). Whether or not the relationship between TL and DNAmAge will persist, or if women with accelerated cellular aging will 'recover' and return to more age-typical levels remains an open question.

We found evidence for CoR using both TL and DNAmAge, yet these two measures of cellular aging appear to reflect different biological pathways linking reproductive effort with senescence. Congruent with this interpretation is the observation that TL and DNAmAge measured in the same individuals have been independently associated with aging and mortality in prior studies (Belsky et al., 2018; Marioni et al., 2016), and capture distinct dimensions of cellular aging (Horvath & Raj, 2018; Lowe et al., 2016; Lu et al., 2018). Accordingly, TL and DNAmAge acceleration were not associated with each other in this study. Accelerated TL attrition – a measure of 'mitotic age' that is modified directly by cellular division – could stem from factors that modify cellular proliferation rates, such as the elevated inflammation, blood cell production, and cell-turnover rates that characterize pregnancy in this and other samples (Kuzawa, Adair, Borja, & Mcdade, 2013; Soma-Pillay et al., 2016).

In contrast to TL, Horvath's DNAmAge is not considered a marker of mitotic age. In vitro DNAmAge is associated with cell passage number, but only in conjunction with the expression of the Telomerase Reverse Transcriptase (*TERT*) gene (Lu et al., 2018), and DNAmAge tracks chronological age even in immortal, non-dividing, and non-

proliferative tissues and cells (Horvath, 2013). Although the biological significance of DNAmAge is unknown, it is hypothesized to reflect the integrity of an epigenetic maintenance system, itself responsible for maintaining dynamic regulatory stability within cells (Horvath, 2013). In light of the hypotheses about the functional underpinnings of DNAmAge, our findings are consistent with the prediction that reproduction comes at a cost of 'maintenance' – in this case at the scale of cellular regulatory maintenance. Exactly how gravidity might lead to DNAmAge acceleration is unclear, but tradeoffs between protein homeostasis and epigenetic control arising from immune activation or the buffering of oxidative stress are plausible pathways (Feder & Hofmann, 1999; Marshall & Sinclair, 2010; Okada, Teramura, & Takahashi, 2014; Ryan, Brownlie, & Whyard, 2016). Indeed, cumulative changes in immune cell composition during pregnancy likely contribute to DNAmAge acceleration with gravidity, although the measure of DNAmAge used here is remarkably robust across tissue types (Horvath, 2013). Nevertheless, the fact that the functionally-distinct measures of TL and DNAmAge show similar associations with gravidity provides strong support for our prediction that reproduction accelerates cellular aging and organismal senescence, at least among the young adult women represented by our sample.

Contrary to our prediction that the costs of reproduction would be greatest among individuals with limited resources (Dribe, 2004; Lycett et al., 2000; Tracer, 1991), we found no evidence for an interaction between gravidity and SES in models predicting either TL or DNAmAge acceleration. While women in low SES conditions in our study very likely experience constraints in energy or nutrient availability, it is still unclear to

what extent SES adequately captures limitations in the resources most relevant to CoR. Given the relatively young age of the participants, however, it is possible that the moderating effect of resource limitation will emerge at more advanced ages. SES in this population may also index factors other than resource availability that contribute to accelerated aging, such as less healthful diets or decreases in physical activity. This does not appear to be a major confounding factor, however, as neither TL or DNAmAge were significantly associated with SES in our models.

Importantly, neither measure of cellular aging obtained in 2005 predicted gravidity over the subsequent 4 years (2005-2009). This suggests that the women in our study are not altering their reproductive output based on their future prospects of health and survival, nor in response to separate physiological or environmental factors also capable of accelerating cellular aging. This runs counter to a life-history framework whereby 'pace-of-living' as captured by TL and DNAmAge is itself predictive of future fecundity (Nettle, 2010; Williams, 1966b).

Intriguingly, currently pregnant women exhibited significantly 'younger' DNAmAge. This finding could reflect the suite of immunological and physiological shifts that occur during pregnancy, including changes in immune cell composition and elevated estrogen levels. At least in some contexts, estrogen can lower oxidative stress (Behl et al., 1997), and elevated estrogen is protective for both TL and DNAmAge (Levine et al., 2016; Yeap et al., 2016). Pregnancy status and accompanying changes in cell composition may therefore be an important confounder to include in future studies investigating the costs of reproduction in women.

Our findings should be considered in the context of several limitations. First, while we attempt to control for socio-ecological factors that could affect both gravidity and our markers of cellular aging, residual confounding arising from differences in health and/or resources remains a possibility. Although the effects were modest, confounding could help explain the slight decrease in effect size of gravidity after adjusting for SES in models 2 and 6. Future studies employing longitudinal measures of TL and DNAmAge acceleration would minimize the potential effects of such confounders (Noordwijk & Jong, 1986), while modeling lactation and other indices for reproductive effort will be necessary for a more complete estimate of the CoR (M. Gurven et al., 2016; Samuli Helle, 2017). Finally, the women in this study all fall within a relatively narrow age range in young adulthood (20-22 years old). Because both TL and DNA-methylation change more rapidly early in adulthood (Frenck et al., 1998; Horvath, 2013), it is possible that both measures are particularly sensitive to reproduction at this time. This leaves open the possibility that the relationship between gravidity and cellular aging is transient – and measurements of TL and DNAmAge later in life will prove important for resolving this question.

In sum, our study suggests that gravidity predicts shorter telomeres and epigenetic age acceleration, measures of mitotic and non-mitotic aging, respectively, among the young women in our sample. The consistency in relationships between gravidity and aging in two distinct pathways—one reflecting cellular turnover, and the second a putative marker of epigenomic regulation—support a cost of reproduction from pregnancy in humans.

Chapter 4. Genome-wide DNA-methylation in blood varies with reproductive status and supports costs and benefits of reproduction in women

Abstract

Women's reproductive history is associated with life expectancy and differential susceptibility to certain cancers, cardiovascular disease and autoimmune disorders. To explore the potential links between the molecular and physiological changes that occur during pregnancy and breastfeeding and women's health, we examined DNA methylation (DNAm) in a cross-sectional cohort of young (20-22-year-old) women of differing reproductive statuses. Compared to currently non-reproductive women, both pregnancy and breastfeeding were associated with differences in leukocyte DNAm (828 and 1107 loci, respectively). Differences in DNAm were highlighted in genes and pathways related to metabolism, immune function, and cognitive function, all consistent with disease risks associated with reproductive history in women. Most individual loci did not differ between nulliparous and parous (but not currently pregnant or breastfeeding women), hinting at largely transient effect of pregnancy and breastfeeding on the methylome. However, nulliparous and parous women differed in blood leukocyte composition. Furthermore, the top-ranking differences in methylation, though not significantly different between these groups themselves, were enriched for several biological processes that hint at potentially durable impacts of reproduction on women's biology. Although cross-sectional, our results point to several genes and pathways that could explain the link between reproductive

history and women's health later in life, motivating further research employing a longitudinal design.

Introduction

Tradeoffs are a foundational concept in evolutionary biology and a core principle in evolutionary medicine (Grunspan, Nesse, Barnes, & Brownell, 2018). Tradeoffs occur when improvements in one function or trait come at the expense of another and arise due to functional constraints and the finite nature of time and the ability to acquire and allocate energy or other resources (S. C. Stearns, 1989; Williams, 1966a). The tradeoff that occurs between two such demands – reproduction and somatic maintenance – underlie what are referred to as 'costs of reproduction' (CoR) (Williams, 1966b).

In human females, evidence for CoR in the form of disease susceptibility and shortened lifespan comes from both historical records and epidemiological studies. Historical records have shown that the total number of children borne over a woman's lifetime negatively predicts post-menopausal lifespan among frontier populations in Quebec and Utah (1599-1870) (Gagnon et al., 2009), as well as among the British Aristocracy (740-1867) (Westendorp and Kirkwood, 1998). Similarly, mothers of twins in pre-industrial Finland died of infectious disease at higher rates than mothers of singletons, especially when they began their reproductive careers early (S. Helle, Lummaa, & Jokela, 2004). While not all historical studies uniformly support for CoR (Le Bourg, 2007), retrospective approaches using historical records can only address factors that were recorded at the time. This rarely includes key variables related to resource availability (i.e.

nutrition, living conditions) or the health-associated biological processes that might underpin CoR (but see S. Helle et al., 2004; Lycett et al., 2000).

Epidemiological studies among contemporary populations address some of the limitations of historical records and provide additional support for CoR in women. Numerous large studies and meta-analyses across multiple countries demonstrate positive relationships between parity and the risk for cardiovascular disease-related mortality (W. Li, Ruan, Lu, & Wang, 2019; Lv, Wu, Yin, Qian, & Ge, 2015), kidney cancer (Guan, Wu, & Gong, 2013), and all-cause mortality (Grundy, 2009; Grundy & Tomassini, 2005; Tamakoshi et al., 2011; Y. Zeng et al., 2016; Grundy & Kravdal, 2008). Studies also suggest that mortality from diabetes, cancer of the uterine cervix, gallbladder disease, kidney disease, and hypertension are higher among parous relative to nulliparous women (Beral, 1985). Even late-life cognitive decline and Alzheimer's Disease risk have now been linked to parity (Beeri et al., 2009; but see M. Fox, Berzuini, Knapp, & Glynn, 2018), possibly through well-documented changes in both brain structure and function during pregnancy (de Lange et al., 2019; Glynn, 2010; Hoekzema et al., 2017).

Costs of reproduction on women's health have thus been widely supported by both historical and contemporary epidemiological records. However, these findings have not been as well-supported by an array of anthropometric, physiological, or cellular measures. Higher parity is associated with lower hemoglobin levels among women in Kenya and Tibet (Cho et al., 2017; E. M.

Miller, 2010), but not among Tsimane women in Bolivia (M. Gurven et al., 2016). Tsimane women also showed no significant changes in several other measures of immune function and only transient changes in indicators of nutritional status with measures of reproductive investment (M. Gurven et al., 2016; but see Stieglitz et al., 2015). At the cellular level, measures of DNA oxidative damage and defense have been linked to parity in some populations (Ziomkiewicz et al., 2016), but not others (Ziomkiewicz et al., 2018). Similarly, telomere length – which shortens with cell turnover and senescence – has been positively (Barha et al., 2016), negatively (Pollack, Rivers, & Ahrens, 2018; Ryan et al., 2018), and not at all associated (Lane-Cordova et al., 2017) with measures of reproductive investment. This suggests that current models of costs of reproduction in women – and possibly the measures currently in use to study them – may not capture the pathways that link reproductive investment to women's health later in life.

One approach with the potential to shed light on novel pathways underlying CoR in women may be through epigenetic processes. Epigenetic processes are a collection of biochemical processes involved in regulating gene activity, cell fate, and biological memory (Allis et al., 2015). One category of epigenetic processes – DNA methylation (DNAm) – reflects lasting changes to gene regulation, but may also be malleable over the timescales of pregnancy and breastfeeding, possibly relevant to the 'biological embedding' of tradeoffs and CoR. Furthermore, DNAm is sensitive to many of the physiological changes thought to underlie CoR in women. We recently found evidence that DNA methylation age

(Horvath, 2013) – a measure of somatic maintenance – was accelerated with gravidity among young adult women in the Philippines (Ryan et al., 2018). Similar findings were recently reported in a larger US population (Kresovich, Harmon, et al., 2019), supporting the possible role for DNAm in CoR. We also found evidence that DNA methylation age declined during pregnancy and increased during lactation (Ryan et al., 2018). However, this method targets predictable age-related variation in DNAm at only 353 loci (Horvath, 2013), thus it is unclear whether or not differences in DNAm during pregnancy and lactation more broadly might advance our understanding of the genes and molecular pathways potentially involved in CoR. A study of differences in DNAm that accompany reproduction across the methylome will help address this question.

To address this gap, we examined differences in genome-wide DNAm using a cross-section of 392 young (20-22 years old) women of differing reproductive status. By comparing the DNAm in the blood of currently pregnant and breastfeeding women with both nulliparous and parous women, we sought evidence that changes in DNAm accompany reproduction, while also exploring whether such changes persist among parous, but no longer pregnant or breastfeeding women. To make our results more interpretable and comparable to previous studies of CoR, we combined our epigenome-wide association with functional enrichment and network analysis. We hypothesized that reproduction would be accompanied by differences in DNAm in genes and pathways consistent with shifting investment tied to somatic maintenance. Based on

evolutionary theory and epidemiological research we expected these to involve processes tied to metabolism and energy storage, immune function, and genomic stability. We further hypothesized that a subset of these changes would persist in women who are no longer pregnant or breastfeeding. We therefore expected to observe a subset of differences in DNAm between our cross section of nulliparous and parous women – consistent with a lingering 'cost' of reproduction on the epigenome.

Materials and Methods

Participants and Study Design

Data came from the Cebu Longitudinal Health and Nutrition Survey (CLHNS), a birth cohort study in Metropolitan Cebu, Philippines that began with enrollment of 3,327 pregnant mothers in 1983–1984. The original mothers were interviewed in the home during the third trimester of pregnancy, and women and their infants were assessed immediately following birth, and every 2 months for 2 years. Additional surveys were conducted in 1991–1992, 1994–1995, 1998–1999, and 2002. The current study focuses on the birth cohort, who were 20-22 years of age when blood for DNA-methylation was collected in 2005. Rates of refusal during initial recruitment were low (<4%), and attrition in the CLHNS is due primarily to factors related to out-migration (Adair et al., 2011; Perez, 2015). Informed consent was obtained from all participants and data collection was

conducted under conditions of written informed consent and with oversight by the Institutional Review Boards of the University of North Carolina at Chapel Hill and Northwestern University.

A total of 392 women were included in the current study. These women were drawn from a subsample of 1759 women who provided a blood sample in 2005 and later participated in a pregnancy tracking study (Thomas W. McDade, Borja, Largado, Adair, & Kuzawa, 2016). Reproductive histories were based on an in-home survey administered by a trained interviewer in 2007. The survey included questions about each known pregnancy, its duration, prenatal care, birth outcome (e.g. live birth, miscarriage, stillbirth, twins), and breastfeeding initiation and termination. Date of conception was inferred based on pregnancy duration and date of pregnancy termination (i.e. birth, miscarriage, etc.). For the few occasions when participants could not recall the day of pregnancy termination, the 15th of the month was used. Based on these records, women were classified as pregnant, breastfeeding, parous (but not breastfeeding or pregnant) and nulliparous. Women were classified as ‘pregnant’ when the blood sample date fell between the date of conception and the date of pregnancy termination. Women were classified as ‘breastfeeding’ when blood sample date fell between the initiation of breastfeeding and the termination of breastfeeding. Women with pregnancies prior to the date of blood sample, but who were not otherwise breastfeeding or pregnant were classified as ‘parous’. Women who reported never having been pregnant for any duration up to and during the time of the

blood sample were classified as ‘nulliparous’. Two women who were simultaneously pregnant and breastfeeding were reclassified as ‘pregnant’.

DNA methylation

Overnight fasting blood samples were collected into EDTA-coated vacutainer tubes, centrifuged to separate plasma and white blood cells, and frozen at −70°C. Samples were express shipped to the US on dry ice, stored frozen at −80°C prior to DNA extraction (Puregene, Gentra), and stored at −80°C following extraction. 750ng of genomic DNA was treated with sodium bisulfite (Zymo EZDNA, Zymo Research, Irvine, CA, USA), and 160ng of converted DNA was applied to the Illumina HumanMethylation450 Bead Chip under standard conditions (Illumina Inc., San Diego, CA). Technicians were blind to any information regarding participant characteristics, and samples were randomly assigned to plate, chip, and row. Background subtraction and color correction were performed using Illumina Genome Studio with default parameters. Data were then exported into R for further analysis.

Quality control was performed as part of a larger sample to confirm participant sex and replicate status, and probes for sex chromosomes were removed from further analysis. Probes associated with known single nucleotide polymorphisms (SNPs), unreliable probes with a detection p-value above 0.01, probes with fewer than three beads contributing to signal, and those previously shown to bind to multiple genomic regions (E. M. Price et al., 2013) were also removed, leaving

434,728 probes. Data were quantile normalized using the R *lumi* package, then probe types were normalized using the SWAN method (Maksimovic, Gordon, & Oshlack, 2012). Next, plate, row, and chip batch variables were assessed using PCA and corrected using the COMBAT function in the *sva* R package (Leek, Johnson, Parker, Jaffe, & Storey, 2012). Spearman correlations for one sample run in duplicate and one sample run in quadruplicate all exceeded 0.99, confirming the efficacy of positional and batch effect corrections. Finally, proportions of blood cell types were predicted using a previously established algorithm, and variance associated with cell composition was removed using a linear regression approach (Houseman et al., 2012b; Jones, Islam, Edgar, & Kobor, 2015).

Statistical Analyses

A total of 434,728 probes passed quality control procedures. However, many DNAm sites are largely invariable between individuals and therefore unlikely to be informative with respect to reproductive status or CoR (Mill & Heijmans, 2013; Rakyan, Down, Balding, & Beck, 2011). To concentrate our analyses on sites plausibly associated with reproductive status and to reduce the burden of multiple comparisons (Bourgon, Gentleman, & Huber, 2010), we filtered out probes for which variability in β-values between the 10th and 90th percentiles in our population was <5%. This left us with a subset of 110,631 probes, which were converted from β-values to M-values prior to statistical analyses (Du et al., 2010). For hypothesis testing, probe-wise variance was determined by fitting

linear regression models and applying parametric empirical Bayes smoothing formula over the entire array dataset that passed quality control using the R bioconductor package *limma* (Ritchie et al., 2015). This approach allowed for gene-wise information borrowing to better estimate the variation for each probe. The model outcomes from the subset of variable probes described above were then extracted and corrected for multiple comparisons using the method of Benjamini and Hochberg (1995). The following contrasts were made: nulliparous-pregnant, nulliparous-breastfeeding, parous-pregnant, parous-breastfeeding, nulliparous-parous.

To control for possible confounding social and environmental factors known to affect DNAm (Thomas W. McDade et al., 2019; Zeilinger et al., 2013) we included smoking (current smoker = 1), and a composite measure of socioeconomic status (SES) for both the year the blood sample was taken and the year the participant was born. SES was measured as a combination of income, education, and assets. Participants reported their annual income from all sources, including in-kind services, and the sale of livestock or other products by household members during the prior year, which were summed to determine total household income. Incomes were deflated to 1983 levels, and log-transformed. Participants also reported on maternal education (in years) and nine assets (coded 0, 1) that were selected to capture population-relevant aspects of social class (including electricity, televisions, refrigerators, air conditioners, tape recorder, electric fans, jeepneys, cars, and home and property ownership). In

addition, house construction type (i.e., light, mixed, permanent structure) was coded as 0, 1, and 2, respectively. Thus, asset scores ranged from 0 to 11. A principal components analysis was run on log income and assets at birth (1983) and at sample collection (2005) along with maternal education in Stata (v. 14.1). The first component of variation accounted for 49% of the variation and individual scores for this component of variation were used as our measure of SES.

Finally, to control for the possible confounding influence of population stratification and some of the known impact of genetic variation on DNAm, we obtained genome-wide SNPs using the Global Screening Array (Infinium Global Screening Array-24 v2.0—Illumina). After standard SNP quality control, we performed multidimensional scaling using Euclidean distance (*cmdscale* function in R) to condense SNP variability into two components representing maximal dissimilarity in the data which were used as covariates.

Gene Ranking, Functional Enrichment and Network Construction

Delta betas ($\Delta\beta$) used for ranking the top hits were determined from effect sizes of reproductive status from simple linear regression on untransformed β-values. Gene annotation for each probe was determined using the Illumina annotation UCSC_RefGene_Name column, resulting in some probes being associated with multiple transcripts of the same gene or multiple genes (Hansen, 2015). Using the Illumina-annotated USCS_RefGene_Name annotation we gave each gene analyzed a score. Scores were comprised of the negative log of the minimum

uncorrected differential methylation p-value and the log of the absolute maximum delta beta associated with the gene. The -log10 p-value and log10 delta beta values were then standardized (value-mean/standard deviation). The average of the standardized p-value and delta beta scores was the overall score for that gene. These gene scores were used for the ranking used in Table 3 and functional enrichment ranking of gene ontology (GO) terms.

GO annotations of the 17,303 annotated genes associated with the variable 450K probes used in differential methylation analysis were used as the background list. Enrichment of GO terms in the ranked list of differentially methylated genes was tested using the receiver operator characteristic (ROC) method from ErmineJ (Gillis et al., 2010). The ROC method is based on ranking of gene scores, and enrichment for a gene set occurs when the probes in the examined genes rank higher than expected by chance. Parameters were set as follows: biological process GO terms only were included, 5–100 gene set sizes, and best scoring replicates using the standardized negative log10 p-values and standardized delta betas described above. As a result, it is possible to observe significant enrichment even when there are no differentially methylated sites within a given gene.

Statistical significance is reported as false discovery rates computed using the Benjamini–Hochberg method in ErmineJ. Also calculated are the multifunctionality scores of the ontology gene sets (Gillis & Pavlidis, 2011). When investigating the related functions for each gene, genes were linked to function,

expression, enhancer networks, and disease risks using openly-accessible compendia and curated databases (Fishilevich et al., 2017; Rappaport et al., 2017; Stelzer et al., 2016; Uhlén et al., 2015). Genomic features were based on UCSC genome annotations (UCSC_REFGENE_GROUP) provided by Illumina. The relationship between a CpG and repetitive DNA was derived from the overlap between the intended alignment of the probe and repetitive sequences from RepeatMasker (http://www.repeatmasker.org; RepeatMasker, Institute for Systems Biology, Seattle, WA, USA) and Price et al. (E. M. Price et al., 2013). The number of repetitive basepairs in a probe were categorized as low (<10), medium (>10 and < 40), or high (>40).

Network construction from enriched biological processes was based on the ranked ErmineJ output. First, a gmt file containing GO group terms and associated genes was created using annotation downloaded from (http://download.baderlab.org/EM_Genesets/February_01_2018/Human/). From this list, genesets with an adjusted p-value threshold (FDR Q value ≤ 0.1) were used to construct networks. Networks were generated using the EnrichmentMap application in Cytoscape with the additional parameters: p = 0.05, overlap coefficient 0.5 (Merico et al., 2010; Shannon, 2003).

Results

Descriptive Statistics

A total of 392 women were included in the study. A breakdown by reproductive category is provided in Table 4-1. Women of different reproductive statuses did not differ in age or genetic PC-scores 1 and 2 (P = 0.43 and 0.68, respectively), but did differ by smoking status (P = 0.007) and SES (Table 4-1). While no pregnant or breastfeeding women smoked, three nulliparous women and seven parous, but no longer pregnant or breastfeeding women reported smoking. SES was higher among nulliparous compared to all other reproductive categories ($F_{3,388}$ = 3.63, P = 0.0132). Using hierarchical clustering by distance for the subset of all 110,631 sites examined did not reveal any grouping by SES quartiles, smoking, or genetic PC-score quartiles.

Table 4-1. Descriptive statistics of 392 young (20-22 years old) women of varying reproductive statuses participating in the Cebu Longitudinal Health and Nutrition Survey (CLHNS).

	Nulliparous (N=176)	**Pregnant (N=69)**	**Breastfeeding (N=60)**	**Parous (N=87)**	**Total (N=392)**	**p-value**
Age (yrs)						0.636[1]
Mean (SD)	21.65 (0.35)	21.66 (0.33)	21.72 (0.32)	21.67 (0.36)	21.67 (0.35)	
Range	20.84 - 22.44	20.90 - 22.42	21.05 - 22.47	20.88 - 22.40	20.84 - 22.47	
SES						0.013[1]
Mean (SD)	0.05 (1.35)	-0.50 (1.31)	-0.38 (1.50)	-0.34 (1.41)	-0.20 (1.40)	
Range	-2.75 - 3.95	-2.75 - 3.19	-3.27 - 3.81	-3.10 - 3.90	-3.27 - 3.95	
Genetic PC-Score 1						0.423[1]
Mean (SD)	0.16 (9.01)	-0.42 (8.82)	1.93 (7.55)	0.10 (7.75)	0.32 (8.50)	
Range	-19.94 - 24.04	-21.15 - 15.84	-12.54 - 22.85	-24.95 - 18.70	-24.95 - 24.04	
Genetic PC-Score 2						0.681[1]
Mean (SD)	-0.20 (8.06)	0.46 (7.93)	-0.30 (7.47)	0.91 (6.94)	0.15 (7.70)	
Range	-20.48 - 20.33	-17.07 - 17.50	-15.41 - 17.56	-17.92 - 19.17	-20.48 - 20.33	
Smoking Status (1 = "yes")						0.007[2]
Number (%)	3 (2%)	0	0	7 (8%)	10 (3%)	

[1.] Linear model ANOVA
[2.] Fisher's Exact Test for Count Data

Genome-wide DNAm by reproductive status

After correcting for blood cell composition and the false discovery rate, we detected differences in DNAm with reproductive status in a total of 2120 (1.9%) of the 110,631 sites we examined. These were not evenly distributed across the genomic features we examined (χ^2 (6, N = 110,631) = 203.75, P = 3.02e-41), with fewer than expected differentially methylated positions (DMPs) in unclassified intergenic regions and transcription start sites (both TSS1500 and TSS200) and more than expected DMPs in gene bodies, 3'UTRs and 5'UTRs. The 2120 significant DMPs did not differ in repetitive sequence from the total subset of sites we examined (χ^2 (2, N = 110,631) = 2.76, P = 0.251).

Compared to nulliparous women, differential methylation among pregnant women was observed in a total of 828 CpG loci spanning 533 annotated genes (CpG/gene - range: 1-19, median = 3). Of these 828 DMPs, 96% (795/828) had lower levels of methylation during pregnancy (Table 4-2; Fig. 4-1A). DMPs between pregnancy and nulliparity occurred less often than expected in intergenetic regions and upstream transcription start sites (TSS1500), and more often than expected in gene bodies and 3'UTR (χ^2 (6, N = 110,631) = 138.60, P = 1.97e-27). Compared to the subset of CpG sites analyzed, the DMPs did not differ in the number of repetitive base pairs in the region (χ^2 (4, N = 110,631) = 4.75, P = 0.314).

Compared to parous – but currently non-pregnant or breastfeeding – women, 539 CpG loci were differentially methylated among pregnant women, spanning

352 annotated genes (CpG/gene - range: 1-25, median = 1). Of these 539 DMPs, 99% (533/539) had lower levels of methylation during pregnancy (Table 4-2; Fig. 4-1B). Roughly half (49%, 264/539) of the DMPs when comparing pregnant and parous women overlapped with the comparison between pregnant and nulliparous women (Fig 4-2). DMPs between parous women and pregnant women occurred less often than expected in intergenic regions, and more often than expected in 5'UTR and gene bodies (χ^2 (6, N = 110,631) = 56.64, P = 2.17e-10). Compared to the subset of CpG sites analyzed, the DMPs comparing parous with pregnant women did not differ in the number of repetitive base pairs in the region (χ^2 (4, N = 110,631) = 0.90, P = 0.925).

Compared to nulliparous women, differential methylation among currently breastfeeding women was observed in a total of 1107 CpG loci in 849 annotated genes (CpG/gene - range: 1-6, median = 1). Only 8% (90/1107) of DMPs found among breastfeeding women overlapped with DMPs noted above as associated with pregnancy (Fig. 4-2). In contrast with pregnancy, breastfeeding was associated with higher levels of methylation relative to nulliparity, with 71% (787/1107) of DMPs being more methylated among breastfeeding women. Differences between breastfeeding and nulliparity occurred less often than expected in intergenic regions and nearby transcription start sites (TSS200) and more often than expected in 5'UTRs, gene bodies, and 3'UTRs (χ^2 (6, N = 110,631) = 90.81, P = 2.05e-17). DMPs were not evenly distributed across categories of repetitive sequence, with hypomethylated DMPs occurring more

often than expected in probes with high and medium levels of repetitive sequence (χ^2 (4, N = 110,631) = 22.73, P = 1.44e-4).

Table 4-2. Number of differentially methylated positions (DMPs) among women varying in reproductive status. Numbers are in contrast to the (top) reference level (i.e. Null = nulliparous as reference group).

	Null-Pregnant	Parous-Pregnant	Null-Breastfeeding	Parous-Breastfeeding	Null-Parous
Down	795	533	320	0	0
Non-sign.	109803	110092	109524	110630	110631
Up	33	6	787	1	0

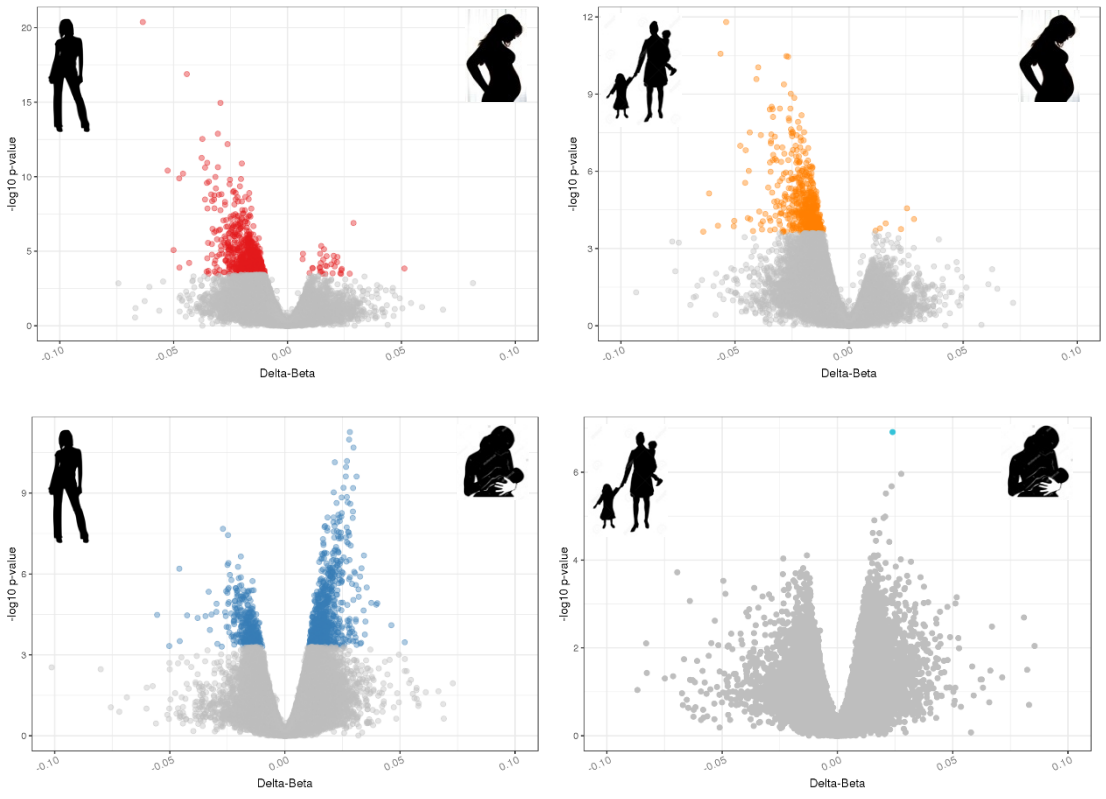

Figure 4-1. Volcano plots of differences in DNAm (Delta-Beta) by negative log10 p-value between women of differing reproductive status. Comparisons between

nulliparous-pregnant (A), parous-pregnant (B), nulliparous-breastfeeding (C), and parous-breastfeeding (D), are shown. Y-axis scale varies by plot.

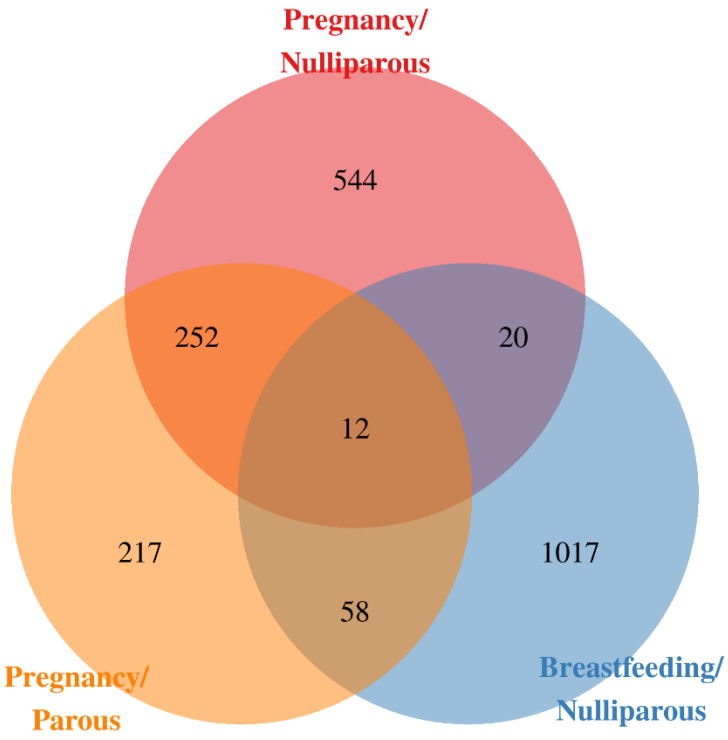

Figure 4-2. Overlapping significant differences in DNAm between women of differing reproductive status. Number of overlapping significant differences shown in black. Single significantly different site for breastfeeding-parous women not shown, but overlapped with breastfeeding-nulliparous category.

Between parous and breastfeeding women, only one DMP was observed (Fig. 4-1D). This site (cg07549715) is located in the gonadotropin releasing hormone 2 (*GNRH2*) gene and was one of the 1107 DMPs found between nulliparity and breastfeeding. There were no statistically significant DMPs between nulliparous and parous women after correcting for false discovery rate.

Gene Ranking, Functional Enrichment and Network Analysis

Genes were ranked based on the sum of maximum standardized -log10 p-values and absolute $\Delta\beta$ for each gene, such that the highest ranked genes are those for which p-values were lowest and differences between groups were highest. The top 10 ranked genes for each comparison of reproductive status are provided in Table 3. Nulliparous-pregnant and parous-pregnant overlapped in 7 of their top 10 genes, mirroring the large overlap in CpG loci between nulliparous-pregnant and parous-pregnant women (Table 4-3). The top 10 genes listed for breastfeeding did not overlap with the pregnancy-associated genes. One gene, *DNAH10*, appeared in the top 10 ranked annotated genes for both nulliparous-breastfeeding and parous-breastfeeding (Table 4-3).

Table 4-3. Top 10 genes differing between reproductive status groups scored using the sum of standardized -log10 p-values and absolute $\Delta\beta$ (group differences). Highest ranked genes are those with the smallest p-values and largest differences between groups.

Null-Pregnant		**Parous-Pregnant**		**Null-Breastfeeding**		**Parous-Breastfeeding**	
Gene	score	Gene	score	Gene	score	Gene	score
CLEC2D	10.836	*CLEC2D*	6.972	*DNAH10*	5.700	*GNRH2*	5.741
TNFSF10	8.916	*ZEB2*	6.331	*FAM193B*	5.561	*CPM*	5.044
CUEDC1	7.738	*SBNO2*	5.938	*MLNR*	5.455	*FAM13A*	4.725
CCR7	6.679	*NADK*	5.859	*SLC38A10*	5.162	*ANKFY1*	4.533
SBNO2	6.514	*RORC*	5.623	*AMBRA1*	5.050	*LCP2*	4.094
BMP1	5.859	*NACC2*	5.324	*KIAA0146*	5.024	*DNAH10*	4.053
TMEM49	5.828	*CUEDC1*	5.068	*ASPRV1*	4.966	*ST5*	3.888
NADK	5.717	*GGT6*	4.948	*VTI1A*	4.736	*LSM12*	3.719
CISH	5.688	*TNFSF10*	4.946	*IL1R1*	4.658	*FOXP1*	3.708
ZEB2	5.513	*BMP1*	4.904	*ABCC1*	4.526	*CCRL2*	3.671

Compared with nulliparity, the pregnancy-associated methylome was associated with enrichment for pathways involved in T cell activation, adhesion, and signaling; cellular

responses to interferon-gamma (IFNγ); negative regulation of the viral life cycle; and cell morphogenesis involved in neuron differentiation (Fig. 4-3). Parous women and pregnant women also differed in processes involved in T cell activation and adhesion and exhibited methylomes with enrichment for processes involved in axon guidance, development, and neuron differentiation (Fig. 4-4).

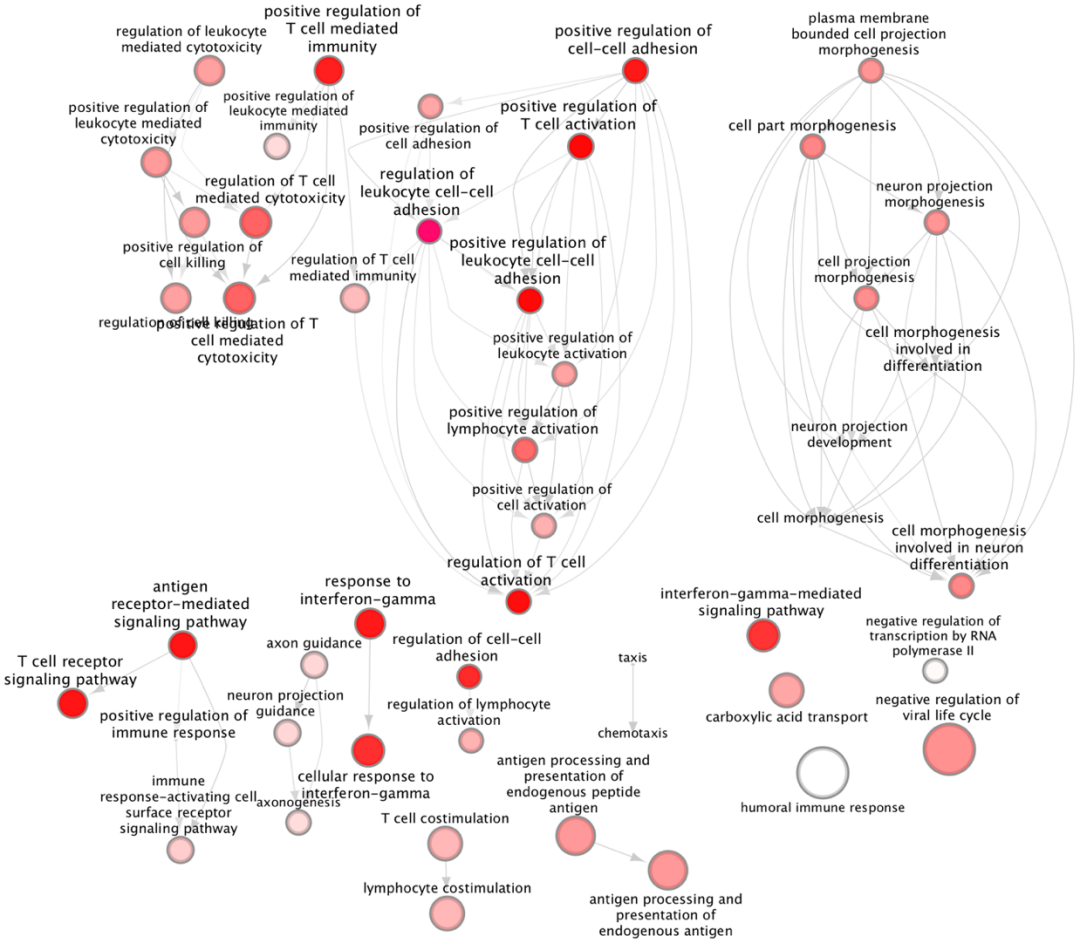

Figure 4-3. Nulliparous – Pregnant Women: Network of enriched biological processes based on differential methylation in pregnant women compared to nulliparous women. Darker colors indicate smaller false-discovery rate (FDR) corrected enrichment p-values based on ranking using the receiver operating characteristic and gene scores. More description in the methods.

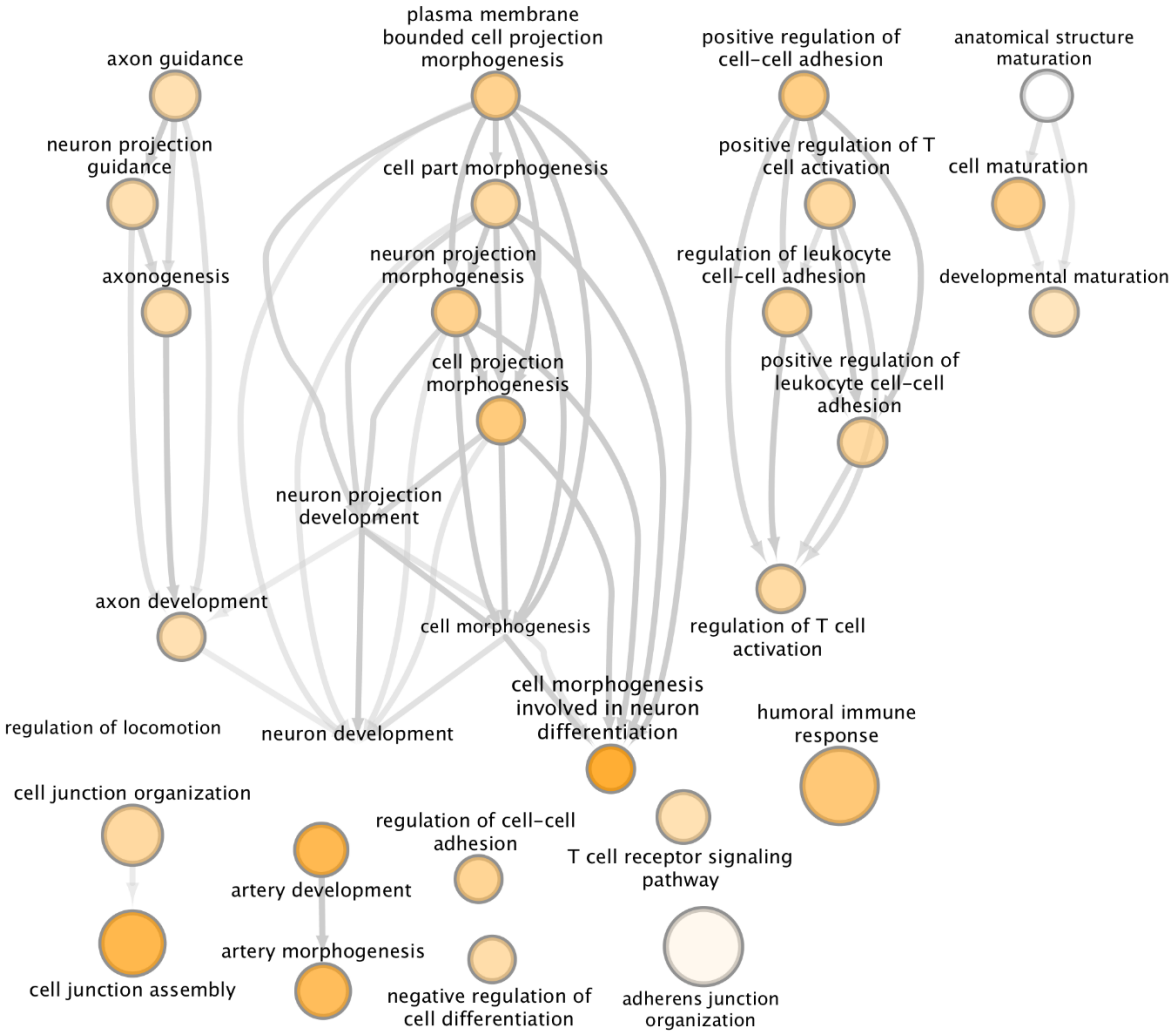

Figure 4-4. Parous – Pregnant women: Network of enriched biological processes based on differential methylation in pregnant women compared to parous women. Darker colors indicate smaller false-discovery rate (FDR) corrected enrichment p-values based on ranking using the receiver operating characteristic and gene scores. More description in the methods.

Compared to nulliparity, the breastfeeding-associated methylome was enriched for nuclear, protein, and vesicle-mediated transport, and for antigen processing and presentation of exogenous peptide antigen via MHC class II (Fig. 4-5). Relative to the

methylomes of breastfeeding women, the methylomes of parous women did not exhibit enrichment for any biological processes.

While no individual CpG loci differed between nulliparous and parous women, the top-ranking genes were significantly enriched for processes involved in axon development, neurogenesis, and cell junction assembly and adhesion (Fig. 4-6).

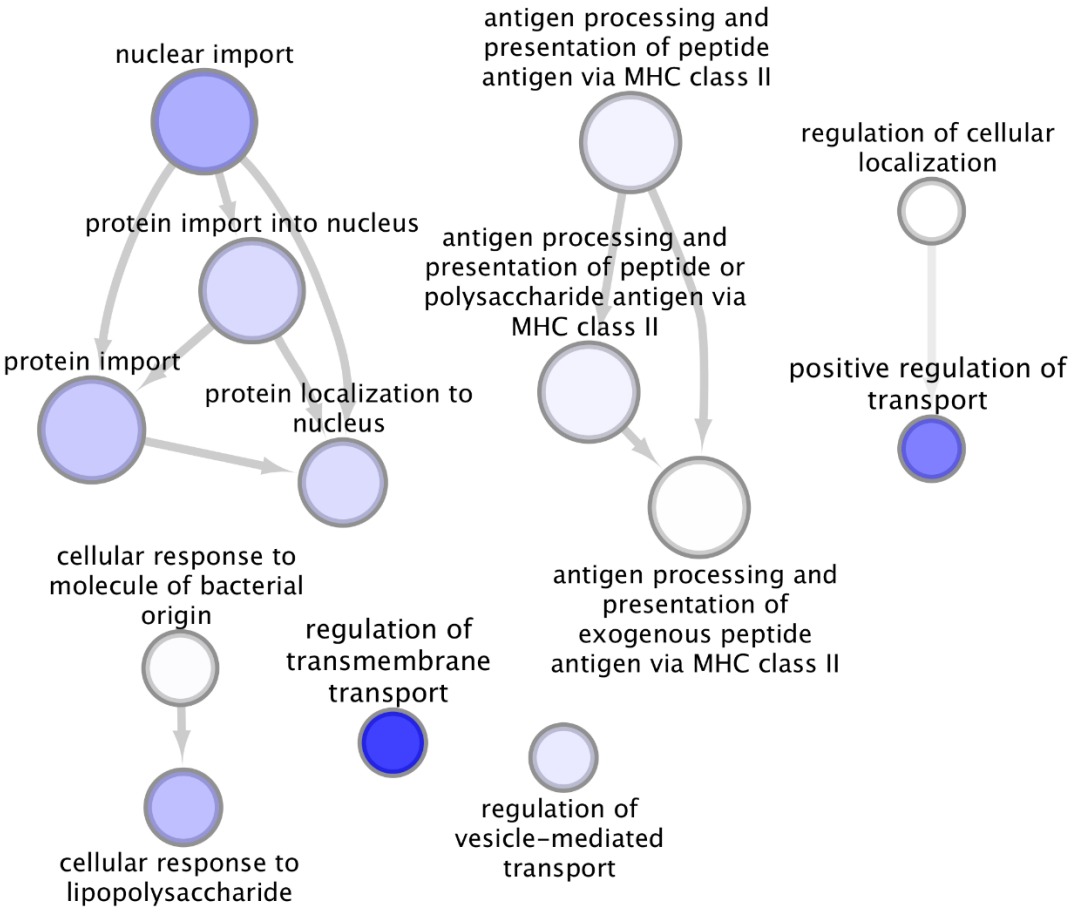

Figure 4-5. Breastfeeding – Nulliparous women: Network of enriched biological processes based on differential methylation in breastfeeding women compared to nulliparous women. Darker colors indicate smaller false-discovery rate (FDR) corrected enrichment p-values based on ranking using the receiver operating characteristic and gene scores. More description in the methods.

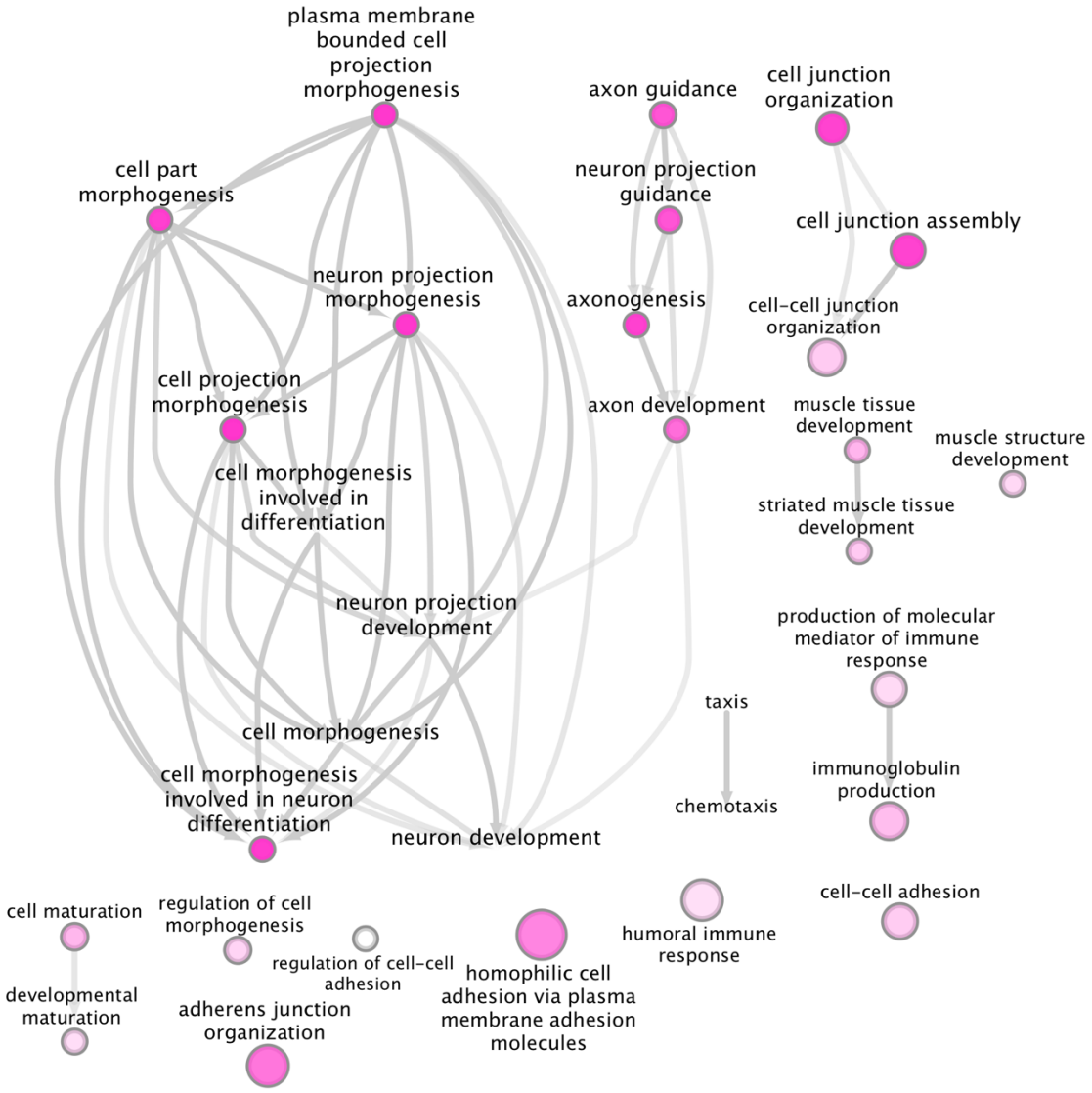

Figure 4-6. Parous– Nulliparous women: Network of enriched biological processes based on differential methylation in parous women compared to nulliparous women. Darker colors indicate smaller false-discovery rate (FDR) corrected enrichment p-values based on ranking using the receiver operating characteristic and gene scores. More description in the methods.

Blood Cell Composition

Our analyses of genome-wide DNAm employ reference-based algorithms that correct for individual differences in immune cell composition in the blood. The differences in

DNAm described above were apparent despite significant differences in imputed cell composition between groups (Fig. 4-7). Nevertheless, differences in cell composition that group by reproductive status may themselves be informative with respect to understanding tradeoffs tied to reproduction in women. For example, we observed significant differences between pregnant and nulliparous women for all cell types (Fig. 4-7). However, for B-cells, CD4T cells, CD8T cells, and granulocytes, these differences were not present between nulliparous and breastfeeding women (Fig. 4-7), suggesting that levels return to pre-pregnancy levels among breastfeeding women. In contrast, cell composition for monocytes and natural killer cells among breastfeeding women was lower and higher, respectively, than among nulliparous women. These significant differences were in the opposite direction to those observed in pregnant women and suggest what might be described as a ‘rebound’ in cell proportions. Surprisingly, these differences were also present among parous women (Fig. 4-7), hinting at durable alterations in systemic immune regulation after pregnancy and breastfeeding.

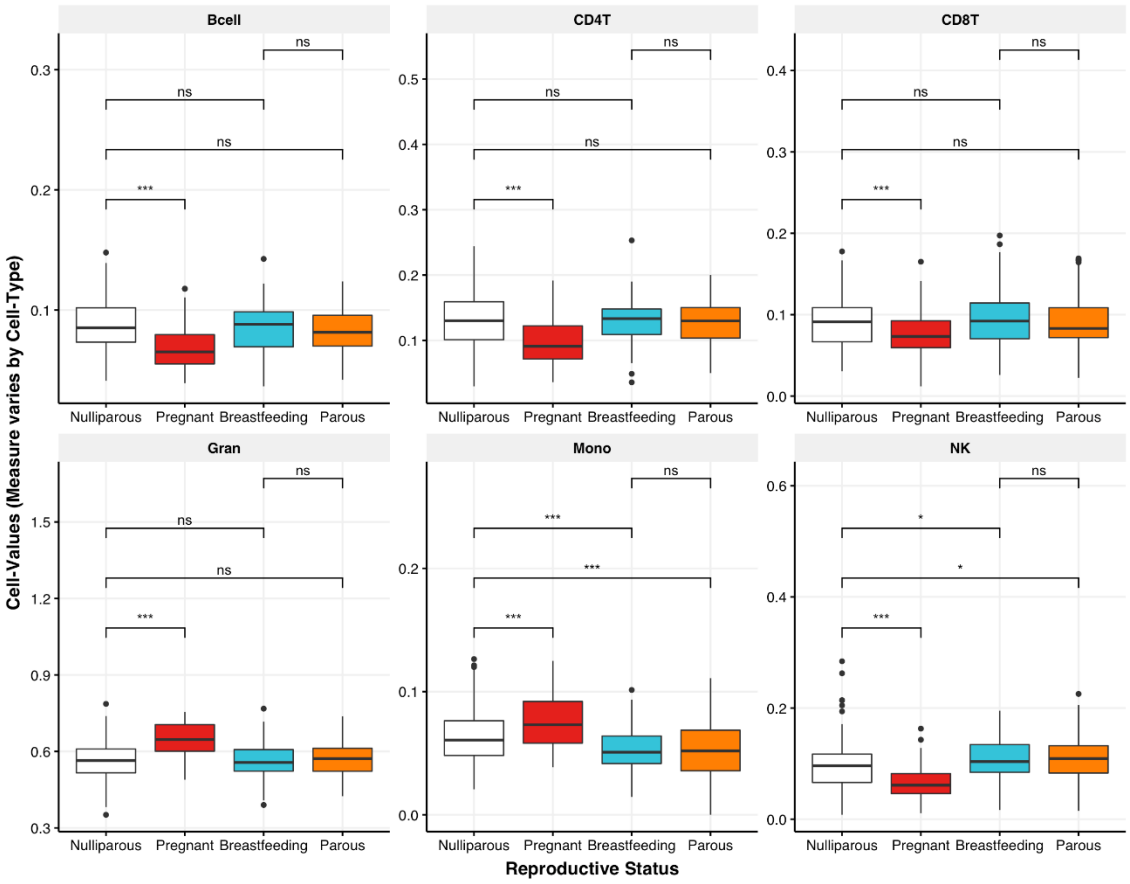

Figure 4-7. Imputed immune cell composition among women of differing reproductive status. B lymphocytes (Bcells), CDT4 lymphocytes (CD4T), CD8T lymphocytes (CD8T), granulocytes (Gran), monocytes (Mono), and natural killer cells (NK).

Discussion

Disease risk and all-cause mortality varies with women's reproductive history. Elevated risk for some diseases and shortened life-expectancy is consistent with energetic or functional tradeoffs tied to pregnancy or lactation, referred to as 'costs of reproduction'. While a range of biological markers have begun to support the broad physiological processes underpinning CoR in women, much remains to be learned about the genes and molecular pathways involved. We used differences in DNA methylation (DNAm) among women of differing reproductive status to shed light on these molecular processes. Our findings indicate that the blood methylome varies markedly with reproductive status. Furthermore, differences in DNAm during pregnancy were almost entirely distinct from those observed during breastfeeding. Our analysis of parous women suggest that these differences are largely transient in blood, yet enriched neurogenesis and adaptive humoral response immune pathways could reflect subtle residual effects of reproduction on women's methylomes. Reproductive status was also associated with significant differences in bioinformatically-derived measures of cell proportions, further suggesting that changes to the immune system may be major factor linking reproduction to women's health.

Pregnancy

Compared to nulliparous women, pregnant women exhibited lower levels of DNAm for over 95% of the observed differentially methylated positions (DMPs). A similar pattern was observed when comparing pregnant women to ever-parous (but not pregnant or

breastfeeding women) where nearly 99% of the DMPs in pregnancy were down-methylated. Although the functional consequences of lower levels of DNAm varies by gene, CpG class (e.g. islands, shores, shelves) and genomic features (e.g. introns, exons, 5'UTR, 3'UTR), global loss of methylation over time is a signature of aging in both humans and other species (Bollati et al., 2009; Jones, Goodman, et al., 2015; Jung & Pfeifer, 2015). The differences in the methylome that ranked the highest were found near genes tied to age-associated phenotypes. Genetic variation near *SBNO2* or its enhancer is associated with bone mineral density (Kichaev et al., 2019), lung function (FEV/FEC) (Fishilevich et al., 2017; GH19J001851) and Alzheimer's Disease risk (GH19J001062), while variation near *ZEB2* is associated with lung function and grip strength (Kichaev et al., 2019; Tikkanen et al., 2018). These physical measures of age-related decline are strongly linked to health, disability, morbidity, and mortality in the elderly (Bohannon, 2008; Sharma & Goodwin, 2006), and variation in DNAm in these genes during pregnancy could provide a link between reproduction and age-related health outcomes later in life. Thus, our findings suggest that pregnancy is characterized by epigenetic signatures of 'aging' across the maternal methylome, but that these differences may be largely transient.

Hypomethylation – typically when associated with retroelements or other repetitive sequences – is also a hallmark of cancer and tumorigenesis (Ehrlich, 2002; Kanai & Arai, 2012). In our sample, pregnancy-associated hypomethylation was not disproportionately found in regions with repetitive DNA (but see below for such findings in during breastfeeding). However, fetal nucleated red blood cells are hypomethylated

relative to maternal leukocytes, hinting at fetal microchimerism through the 'contamination' of maternal blood (de Goede, Lavoie, & Robinson, 2016). Several of the top-ranking genes, however, do have established links to breast cancer. *TNFSF10* (Tumor Necrosis Factor Superfamily Member 10) codes for a protein that binds to so-called 'death receptors' that trigger apoptosis and cell death in tumor cells (Wiley et al., 1995). Furthermore, genetic variation in this gene is a risk factor for estrogen-receptor negative breast cancer (Huo et al., 2016). Cell proliferation in breast cancer also occurs in another top ranking gene *CUEDC1* (CUE domain containing 1) via stimulation of the estrogen-receptor alpha receptor (Lopes et al., 2018), providing a potential genetic link between pregnancy, estrogen-exposure and breast cancer. Such differences in DNAm among genes linked to both ER- and ER+ breast cancer are consistent with a relationship between pregnancy and breast cancer that operates through both estrogen-dependent and estrogen-independent pathways – with the risk for ER- breast cancer mediated in part by breastfeeding practices after birth (Fortner et al., 2019). While these differences are found in blood leukocytes, differences in DNAm in response to changes in estrogen or other pregnancy-associated hormones could provide a surrogate measure of similar changes in mammary or other tissues. The relationship between DNAm in blood and mammary tissue for these genes during pregnancy is unknown, but our research suggests that *TNFSF10* and *CUEDC1* may merit further investigation as molecular intermediates in the link between pregnancy, breastfeeding, and breast cancer risk.

Many of the top-ranking genes associated with pregnancy play key roles in immune function and inflammation. Our findings highlight changes in DNAm associated with broad changes in leukocyte count (*TNFSF10, ZEB2, SBNO2*), and a shift from adaptive to innate immunity (*CLEC2D*) (Astle et al., 2016; Germain et al., 2011). These findings are supported by functional enrichment analysis, which allows us to examine the higher-order biological processes derived from all differentially methylated genes during pregnancy. They are also supported by our bioinformatically-imputed cell counts, which suggest decreased B and T-cell counts, and increased monocyte and granulocyte counts. Enriched processes hinged on the regulation of leukocyte signaling and activation, particularly with respect to T cell activation and the response to interferon-gamma (INF-γ). These processes reflect the transition from a proinflammatory Th1 to an anti-inflammatory Th2 immune environment – previously documented as a normal part of pregnancy and parturition (Trundley & Moffett, 2004). Such shifts in the 'landscape of adaptive immunity' are required for successful implantation by controlling immune intolerance to fetal trophoblast cells, while maintaining active defenses against pathogens (Trundley & Moffett, 2004). Changes in inflammatory profiles during pregnancy have previously been described in this population (Kuzawa et al., 2013; Kuzawa, Fried, Borja, & McDade, 2017), and may have important implications for fetal growth and development. The findings presented here are evidence that changes in the immunological landscape of pregnancy are detectable in leukocyte methylomes, even after applying algorithms to estimate and correct for the proportion of circulating cell types (Houseman et al., 2012b; Jones, Islam, et al., 2015). Future work examining the

relationship between the methylome during pregnancy and birth outcomes may capitalize on these changes to help predict pre-term birth, fetal development, and infant health.

The shift away from acquired immunity described here and elsewhere (E. M. Miller, 2009) may help also explain widely-described reduction in the symptoms or relapse rates for several autoimmune disorders during pregnancy (Morelli, Mandal, Goldsmith, Kashani, & Ponzio, 2015). Accordingly, interactions between *CLEC2D* – the top-ranked gene associated with pregnancy – and the membrane receptor *CD161* are thought to play a role in multiple sclerosis (Germain et al., 2011), the risk and progression of which has been linked to both pregnancy and parity (McCombe, 2018). Furthermore, genetic variation in another high ranking gene, *CUEDC1,* has been linked to glutamate levels – themselves a marker of neurotoxicity and poor prognosis – in patients with multiple sclerosis (Baranzini et al., 2010). Finally, the expression of *CCR7* – another high-ranking gene associated with pregnancy – differs between patients with multiple sclerosis and controls, as well as between patients before and after treatment (Fan et al., 2015). *CCR7* expression, possibly a function of DNAm, may play an important role in the etiology and severity of multiple sclerosis symptoms (Fan et al., 2015). Thus, changes in the regulation of the immune system involving differential methylation in *CLEC2D*, *CUEDC1*, and *CCR7* may provide insights into the molecular relationship between pregnancy and multiples sclerosis (Khashan et al., 2011; McCombe, 2018).

Breastfeeding

Relative to nulliparity and in contrast to the hypomethylation observed during pregnancy, 71% of the differences in DNAm observed during breastfeeding were associated with increased methylation. Promoter hypermethylation is a common feature of cancer, as is the hypomethylation of repetitive DNA. While making up a smaller proportion of DMPs, the hypomethylated DMPs occurred more often than expected in probes containing high to moderate numbers of repetitive base pairs, broadly consistent with an elevated risk for tumorigenesis. One of our top-ranked genes for breastfeeding – *FAM193B* – has also been linked to renal cancer. *FAM193B* is a downstream target of enhancers tied to kidney function and renal disease, and *FAM193B* RNA levels are prognostic of poor renal cancer outcomes (Uhlén et al., 2005). Nevertheless, few of the other highest-ranking genes associated with breastfeeding show clear connections to cancer risk.

In contrast – but consistent with the energetically taxing nature of lactation (Butte & King, 2005) – a number of the highest-ranking genes associated with breastfeeding are involved in energy storage and metabolism. *DNAH10, FAM193B*, and *FAM13A* all have been shown to have relationships with various measures of body mass and composition, triglyceride levels, and insulin resistance (Lotta et al., 2017; Singaraja et al., 2014). *FAM193B* has also been linked to pronounced sex differences in adiposity, which could be especially relevant when studying the relationship between reproduction and obesity among women (e.g. Winkler et al., 2015). Among post-reproductive women, BMI is lower among women who breastfeed compared to those who do not and

decreases with increasing time spent breastfeeding (Bobrow, Quigley, Green, Reeves, & Beral, 2013; Coitinho, Sichieri, & Benício, 2001). The differences in DNAm described here point to the regulation of pathways of energy mobilization during breastfeeding that could affect adiposity and body mass later in life, even after changes in DNAm have returned to their original states.

While energetically costly, breastfeeding also allows mothers to transfer immunological, hormonal, and microbial substrates to neonates. While some proteins and lipids are produced locally in mammary epithelial cells, others appear to be produced elsewhere in the body and transported to breast tissue for eventual secretion in breastmilk (Bardanzellu, Fanos, & Reali, 2017). Such substrates may be transported to milk in free, bound, or enclosed in lipid-vesicles called exosomes (Zhou et al., 2012). We found that, compared to nulliparity, breastfeeding was enriched for antigen processing and presentation via the major histocompatibility complex (MHC) class II, as well as transmembrane and vesicle-mediated transport. MHC class II proteins have been found in breastmilk exosomes and may be a source of passive immunity in infants (Admyre et al., 2007), which may in part explain our findings.

Several of the high-ranking genes that were differentially methylated in currently breastfeeding women are also related to neonatal health and development. *DNAH10* is involved in the force generating capacity of cilia in organs such as the lungs and underlies primary ciliary dyskinesia, a disease which is characterized by a failure to adequately clear fluid from the lungs among neonates (Berg et al., 2011; Rappaport et al., 2017). *MLNR* codes for the motilin receptor, and is expressed in its highest

concentrations in the nerves of the antral walls of the stomach and the smooth muscle of the upper walls of the gastrointestinal tract (Uhlén et al., 2015). There, the motilin receptor plays a role in gastrointestinal contraction and motility (Stelzer et al., 2016). Although do not have evidence that MLNR is transferred to offspring, it is tempting to speculate that the differences that we document in breastfeeding vs. nulliparous women partly reflect the cellular production and packaging of vesicle-bound products destined for breastmilk. Such signaling, possibly through vesicle-bound transcription factors or non-coding RNAs, could provide cues important for infant growth and development. Consistent with this hypothesis, motilin is found in high quantities in the blood of breastfeeding women, as well as in breastmilk, where it may play a role in infant gastrointestinal motility (J. Liu et al., 2004). DNAH10 protein is also found in cow's milk and may also be present in human breastmilk (Affolter, Grass, Vanrobaeys, Casado, & Kussmann, 2010). Thus, *DNAH10*, *MLNR*, and perhaps other genes might play a role in the connection between breastfeeding and infant health and development.

Lactation has known inhibitory effects on menstruation and fertility in women (Peter T Ellison, 1990; McNeilly, Tay, & Glasier, 1994). Through this process – referred to as lactational amenorrhea – mechanical and sensory stimuli associated with breastfeeding interact with energy status to suppress ovulation. This occurs through disruption of the pulsatile hypothalamic release of gonadotropin releasing hormone (GnRH), which disrupts secretion of luteinizing hormone (LH) from the anterior pituitary (McNeilly et al., 1994). LH in turn is necessary for the pre-ovulatory estrogen surge that drives the development of the corpus luteum and ovulation (Plant & Zeleznik, 2014). The top-

ranking gene when comparing breastfeeding women to parous women was *GNRH2* (gonadotropic releasing hormone 2), a closely related gene to *GNRH*. This same site was also differentially methylated when breastfeeding women were compared to nulliparous women.

The finding of differential methylation in *GNRH2* in the blood is somewhat surprising given that expression of this gene is thought to be localized in the brain and reproductive tissues (e.g. ovaries, cervix, and fallopian tubes)(Uhlén et al., 2015). However, concordance between DNAm in blood and brain does exist for certain loci, with variability in DNAm and blood-brain concordance for these DMPs moderate to high (> 0.05 and > 0.35, respectively)(Edgar, Jones, Meaney, Turecki, & Kobor, 2017). Given the well-documented role of GNRH in the lactational amenorrhea that accompanies breastfeeding, these findings hint at the possibility that other brain-associated changes may be detectable in the differential methylation of genes in blood. Consistent with this suggestion, the highest scoring probe for each of the highest scoring genes in the enrichment of 'cell morphogenesis involved in neuron differentiation' (*SRGAP2, PIP5K1C, CAP2*) all have moderately high (spearman's ρ = 0.19-0.37) correlations in DNAm between blood and brain (Table S1), suggesting that a subset of the DNAm differences identified in blood in this study may partly reflect changes to DNAm in the brain.

Another intriguing possibility is that some of the apparent transient changes in DNAm identified in our analyses reflect mammary stem cells reported in human and non-human breastmilk (Witkowska-Zimny & Kaminska-El-Hassan, 2017). Researchers have

shown that mammary stem cells are transferred to the brain of suckling pups in mice, where they are integrated into the brain and differentiate into neuronal and neuroglial cells *in vivo* (Aydın, Yiğit, Vatandaşlar, Erdoğan, & Öztürk, 2018). While the differentiation of maternal mammary stem cells into neuronal cells in the human infant is difficult to demonstrate, researchers have found support for this hypothesis by successfully differentiating human mammary stem cells into neural stem cells, neurons, and neuroglia *in vitro* (Hosseini, Talaei-khozani, Sani, & Owrangi, 2014).

If the multipotent capacity of mammary cells can be harnessed, breastmilk could offer therapeutic potential for a range of neurological disorders – even age-related cognitive decline linked to parity in women (Beeri et al., 2009).

Parous women

When comparing DNAm profiles among parous, but not currently reproductive women to nulliparous women we did not observe a significant difference in DNAm for any individual locus. While our study examines different women cross-sectionally, this finding is most consistent with transient – rather than durable – changes to the methylome. This does not rule out a role for DNAm in women's CoR. Any durable changes in DNAm that accompany pregnancy and breastfeeding may be small, cumulative, and heterogeneous across women, making them difficult to detect. Furthermore, temporary changes in DNAm could have more lasting impacts by affecting the regulation of downstream genes, which will invariably be associated with greater stochasticity between individuals, making it harder to detect using the cross-sectional

design we employed here. Finally, all of the parous women in our study had been pregnant only once prior to the blood sample, while all women who with 2 or more pregnancies were pregnant or breastfeeding at the time of the blood sample. We therefore lack variation for the widely-described cumulative effects of multiple pregnancies (Lv et al., 2015; Y. Zeng et al., 2016).

Despite shortcomings, we do find some evidence for lasting changes in parous women. This is reflected by differences in the immune system of parous women compared to nulliparous women in the proportions of monocytes and natural killer cells. Differences in the proportion of monocyte and natural killer cells between nulliparous, pregnant, breastfeeding, and parous women imply functional changes in the immune system of women who have undergone reproduction – changes which could translate immunological changes relevant to women's subsequent health. Although we found no statistically significant DMPs between nulliparous and parous women, changes in immune response are supported by our enrichment analysis, which relies on non-parametric ranking of p-values rather than a p-value threshold. We found an enrichment for biological processes linked to immune regulation, but also neurogenesis. The latter is consistent with our findings for breastfeeding, which point to processes involved in neurogenesis and axon differentiation, and may relate to epidemiological findings supporting higher risk for Alzheimer's among high parity women (Beeri et al., 2009).

Limitations and Future Directions

We leveraged genome-wide DNAm to study the link between reproduction and women's health from an evolutionary perspective. However, our analyses were restricted to a cross-sectional study of women in different reproductive states, not individual women through time. This limits our ability to make definitive claims about 'changes' in the methylome throughout reproduction. The use of a long-term prospective cohort should attenuate confounding for some factors; all women are the same age and have been studied since before their birth, reducing potential influences of age or secular changes in fertility. However, despite our design, women still differ in health or access to resources, which could affect reproductive decisions and the methylome. For example, nulliparous women had significantly higher SES than women who were in the other reproductive states, which could generate false positives in our comparisons. We attempted to address this statistically by including a composite measure of SES for both the year the blood sample was taken as well as the year the woman was born. We also included comparisons between pregnant or breastfeeding women and parous women, who did not differ in SES from pregnant or breastfeeding women. These two measures – combined with the fact that there were no significant individual DMPs when comparing nulliparous to parous women – support the interpretation that our findings are a result of differences in reproductive status and not SES. This interpretation is also supported by the fact that enrichment for neuron differentiation was observed between nulliparous and parous women (who differed in SES) and between pregnant and parous women (who did not differ in SES). Despite these efforts, and the fact that many of our findings

point to changes in systems known to undergo physiological and immunological adjustment during reproduction, our findings – including those showing differences in cell types between nulliparous and parous women – must be interpreted cautiously. A longitudinal approach, following individual women over time and through reproductive transitions, will be vital to addressing these limitations.

Our analyses on blood make it difficult to infer changes in DNAm in other tissues (e.g. breast, ovaries, thymus, liver). Although some epigenetic changes are consistent across tissues, which DMPs provide reasonable surrogates for which tissues is unclear. Nevertheless, some of our leading genes associated with neurogenesis have been found to be modestly correlated between blood and brain, lending some support for our use of blood for preliminary studies of CoR that may relate to disease in brain and perhaps other tissues (Edgar et al., 2017). Additional research using post-mortem tissues or animal models may help to resolve these questions further.

This study highlights a number of potential genes and pathways that may be important for understanding the relationship between reproduction and women's health. However, at this stage we do not know if the changes in methylation in the genes and pathways we described ultimately lead to differences in health later in life. To confirm this will require long-term studies that examine methylation and health during reproduction and in aging women. Comparing DNAm in the genes described here between women of different disease phenotypes – such as with and without breast cancer – could also lend support to our suggestion that reproduction connects with women's health through changes in DNAm. It is also unlikely that women all respond identically to the metabolic,

immunological, and endocrine changes that accompany pregnancy and breastfeeding. Studies of how underlying genetic variation is involved in women's epigenetic responses to reproduction could help close the gap in our understanding of reproduction, the epigenome, and women's health.

Conclusions

Our findings highlight both well-established and novel genes and pathways in the connection between reproduction and women's health. These processes may help clarify mechanistic underpinnings involved in costs of reproduction in women. They may also shed light on the complex and countervailing forces that influence how the female body has come to adapt to – and even benefit from – reproduction. Both tradeoffs and adaptations are likely to be important in understanding the constraints and evolutionary processes that shape women's health and reproduction.

Chapter 5. Testosterone levels are not associated with individual changes in DNA methylation or bioinformatically-imputed cell composition, but do predict epigenetic age in a small sample of young (20-22 year old) Filipino men

Abstract

Testosterone (T) is a steroid hormone responsible for male sexual differentiation and secondary sex characteristics. T also underpins behavioral and somatic components of male mating effort in adulthood and thus contributes to reproductive success. Research suggests that T may be costly to male health and longevity through behavioral, metabolic, or immunosuppressive effects, but the biological pathways involved have not been well-defined. Furthermore, research in human males has largely focused on the putative immunosuppressive effect of T, but support for this effect has been mixed. This inconsistency may be tied to tradeoffs within the immune system, such as between innate and acquired immunity. Alternatively, other measures of metabolic or cellular aging may be involved. In an effort to address these gaps, we examined the relationship between an epigenetic process known as DNA methylation (DNAm) and three measures of T in 90 young (20-22 year old) men in the Philippines. DNAm can be used to calculate measures of 'epigenetic age', which are highly predictive of metabolic health, cellular aging, and mortality. DNAm can also be used to bioinformatically-imputed leukocyte proportions, which we used to generate an axis of innate-acquired immunity to study the putative effects of T on immune function. Finally, to shed new light on T-associated tradeoffs, we carried out a scan of relationships between T and DNAm across the genome. All measures of epigenetic age were positively associated with all measures of T, although only Levine's DNAmPhenoAge – linked to metabolic health

and mortality – was statistically significant. Neither cell types nor our composite of innate-acquired immunity was associated with any measures of T, inconsistent with a simple immunosuppressive effect of T. None of our epigenome-wide association study findings were significant after correction for false discovery, although one gene linked to IL2 and IL4 cytokine production approached significance for evening salivary T. Our preliminary findings using a relatively small sample size point to potentially complex roles of T in metabolic function and perhaps immunity, and suggest that DNAm – in conjunction with later, more well-powered samples – may provide new ways of operationalizing T-associated tradeoffs in men.

Introduction

Reproduction is thought to contribute to poor health and shortened lifespan in humans and other organisms by drawing energy away from somatic maintenance and repair (Crews, 2003; K. Hill & Kaplan, 1999; Rose, 1994; Williams, 1957). This theory – referred to as the 'disposable soma theory' (Kirkwood, 1977) – is supported in plants, insects, reptiles, birds, and mammals (Ardia, Schat, & Winkler, 2003; Kirkwood & Rose, 1991; Obeso, 2002; Peña et al., 2020; Shine, 1980). Costs of reproduction (CoR) have also been found for humans in that life expectancy is reduced, and biological markers of aging accelerated, among women with high parity (Grundy & Tomassini, 2005; Ryan et al., 2018; Tamakoshi et al., 2011; Ziomkiewicz et al., 2016). CoR in women may arise from the fact that female mammals are burdened with substantial energetic demands during pregnancy and lactation (Emery Thompson, 2013; Prentice & Prentice, 1988). Pregnancy and lactation also entail extensive metabolic, immunological, and endocrinological changes that could work in opposition to a mother's own bodily maintenance (Bigiu & Pandi, 2015; S. Helle et al., 2004).

In contrast with females, male mammals do not invest energy directly into offspring growth and development and do not undergo the dramatic endocrinological and immunological changes required to sustain pregnancy and lactation. Instead, male reproductive effort is thought to consist of securing social status, competing and forming alliances, courtship or coercion, and providing protection, provisions, or social support for family, mates, and offspring (Clutton-Brock, 1989). These traits and behaviors, while not mutually exclusive, are categorized as investments into either mating or parenting

effort (Geary, 2015; Trivers, 1972). Paternal care is thought to be a derived characteristic of the human lineage (Geary, 2015; P. B. Gray & Anderson, 2010) and was likely modest among ancestral humans, who appear to have been highly polygynous at least until the Out-of-Africa bottleneck (Amster, Murphy, Milligan, & Sella, 2020). Compared to males of most other mammalian species, human males invest quite heavily into parenting effort through provisioning and protection. However, the costs of paternal care may be outweighed by direct physiological costs of mating effort, which appear to involve behaviorally, metabolically, and immunologically-costly traits directly or indirectly linked to social status and mate attraction.

In humans, as in reptiles, birds, and other mammals, mating effort is largely regulated through a class of steroid hormone known as androgens, in particular testosterone (T) (Adkins-Regan, 2005; Bribiescas, 2001; Flinn, Ponzi, & Muehlenbein, 2012; R. J. Nelson, 2005). T contributes to the development and maintenance of primary and secondary sex characteristics, including male-typical genitalia, bone and facial structure, body hair, and vocal pitch (E. Nieschlag, Behre, & Nieschlag, 2012). T is also an anabolic hormone that increases bone density and muscle size, metabolic rate and lipolysis, and strength (Bhasin et al., 2012; Vanderschueren, Sinnesael, Gielen, Claessens, & Boonen, 2012; but see Alvarado et al., 2015). In the brain, T has both organizational and activational effects on neural development and behavior (Bancroft, 2012; Trumble, Jaeggi, & Gurven, 2015). T also contributes to – and is produced in response to – competitive interactions in which male social status is threatened (Dreher et al., 2016; Eisenegger, Haushofer, & Fehr, 2011; M. N. Muller, 2017), or in response

to potential mating opportunities (Escasa, Casey, & Gray, 2011; Flinn et al., 2012; Lee T. Gettler, McDade, Agustin, Feranil, & Kuzawa, 2013; P. B. Gray, McHale, & Carré, 2017). Further supporting a role of T in human male mating effort, T is highest among men who are single and childless, with levels declining as they transition to stable pair-bonded relationships and fatherhood (L. T. Gettler, McDade, Feranil, & Kuzawa, 2011; P. B. Gray, Kahlenberg, Barrett, Lipson, & Ellison, 2002).

While elevated T may be an important component of mating effort and fitness, it is also widely postulated to be a source of CoR in males of numerous species, including humans. In men, castration – which all but eliminates endogenous production of T – has been associated with a lengthening of lifespan (Min, Lee, & Park, 2012; but see Eberhard Nieschlag, Nieschlag, & Behre, 1993). More subtly, declines in T that accompany men's transition to pairbonding and fatherhood mirror commonly observed reductions in mortality risk of married fathers relative to single, childless men (for a review, see P. B. Gray et al., 2017). Unmarried and non-cohabitating men have higher T, and die younger than married men – and non-fathers die younger than fathers – from nearly every major cause of death (Franke & Kulu, 2018; Keizer, Dykstra, & J. Van Lenthe, 2012). These findings are not an artifact created by men who engage in dangerous behavior or those in poor health being unable to secure opportunities for partnership or fatherhood in the first place, because similar patterns appear when comparing married men and fathers to men who are separated, divorced, or do not share custody of their children (Hu & Goldman, 1990; Ringbäck Weitoft, Burström, & Rosén, 2004). Elevated causes of death for single men and childless men include

substance abuse, accidents, suicide, and disorders of the circulatory, respiratory, and digestive systems (Franke & Kulu, 2018), suggesting that elevated T could be costly for both behavioral and physiological reasons.

Several pathways that might link T to men's health and longevity have been proposed. These are largely based on theory and data from other species and involve behavioral, metabolic, and immunological processes (Bribiescas, 2001)(Table 5-1). Behavioral costs of elevated T in other species include territoriality, antagonistic interactions, and behaviors that increase predation or accidents (R. J. Nelson, 2005). Territorial and aggressive behaviors may threaten male survivorship through injury exposure to parasites, but can directly benefit mates and offspring through resource access and protection from conspecifics and predators. Conspicuous high-risk behaviors with no obvious purpose can be sexually-selected for if they provide females with 'honest signals' of a male's ability to survive those displays (Fisher, 1930; Folstad & Karter, 1992; Zahavi, 1975). In humans, men engage in certain kinds of risky behavior more often than women, and die more often than women from accidents and homicide (Kruger & Nesse, 2006; M. Wilson & Daly, 1985). Furthermore, single and childless men – who have lower T than married men and fathers – tend to die earlier from substance abuse, accidents, and suicide (Franke & Kulu, 2018). Nevertheless, it is not clear if these behaviors and related morbidity and mortality risks are partly driven by variation in circulating T, in addition to better-understood roles of sociocultural or psychosocial drivers like socioeconomic status, social isolation and depression (Dabbs & Morris, 1990).

Another pathway through which T may give rise to CoR is through its effects on body composition and metabolism (Bribiescas, 2001). T is positively associated with body size (Klimek, Galbarczyk, Nenko, Alvarado, & Jasienska, 2014) and lean muscle mass (Bhasin et al., 2012; Blouin, Boivin, & Tchernof, 2008), which increase resting metabolic rate and daily caloric requirements. As in other species, the high caloric requirements of larger body size and metabolically-active muscle can become a liability in humans when calories or other nutrients are limited, as evidenced by findings that men die sooner than women during extreme food shortages (Grayson, 1993; Zarulli et al., 2018). Energy shortage was likely a powerful selective pressure in human evolution, but tradeoffs between lean muscle mass may be rare in calorically-replete environments most men live in now. While clearly relevant under some contexts, a direct 'cost' of T through higher risk of starvation is unlikely to manifest itself in most contemporary populations. Nevertheless, higher metabolic rate could come at the expense through the production of reactive oxygen species and resulting oxidative damage to proteins, lipids, and nucleic acids (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006).

One of the most commonly studied pathways for CoR in men is through potential pleiotropic effects of T on immunity (Michael P. Muehlenbein & Bribiescas, 2005). Building on early work in birds (Folstad & Karter, 1992), the immunocompetence handicap hypothesis (ICHH) posits that T and its associated secondary sex characteristics provide an honest signal of male mate quality through the immunosuppressive effect of T on immune function (Fisher, 1930; Zahavi, 1975). High-quality males are thought to be sexually selected for their capacity to maintain both

costly T-associated traits and energetically-expensive immune function (M. D. Gurven et al., 2016) necessary to survive pathogens and parasites (Michael P. Muehlenbein, Hirschtick, Bonner, & Swartz, 2010). Broadly consistent with an immunosuppressive effect of T, males suffer less often from autoimmune disorders than females (Olsen & Kovacs, 2002), and appear to be more susceptible to parasites and infectious disease than females (Michael P. Muehlenbein & Bribiescas, 2005). Providing a more direct link to T, androgen receptors – which bind testosterone and other androgens – have been found on B and T-lymphocytes, and androgens have been shown to alter immune cell composition, signaling, cytokine production, and cell death (Michael P. Muehlenbein & Bribiescas, 2005; Olsen & Kovacs, 2002).

Nevertheless, evidence for a relationship between T and measures of immunity in non-human animals has been mixed, and evidence for the ICHH in humans is equivocal (Michael P. Muehlenbein & Bribiescas, 2005). While some studies in human males have demonstrated a negative relationship between androgens and immune response (Prall & Muehlenbein, 2015; Trumble et al., 2016), several others report no relationship (Nowak et al., 2018) or even positive relationships (Lee T. Gettler et al., 2014; Nowak et al., 2018; Prall & Muehlenbein, 2015). Part of the challenge may lie in the fact that ‘phenotypic correlations’ can mask tradeoffs at the level of the population (K. R. Hill & Hurtado, 1996; David Reznick, Nunney, & Tessier, 2000). Consistent with T acting as an honest signal, high quality males with the best prospects can afford to maintain both high T and immune function, while poor quality males must reduce investment in both.

Another explanation for the mixed relationship between T and immune function may arise from tradeoffs in development of the immune system itself (Prall & Muehlenbein, 2015). Innate, non-specific immunity, which includes natural killer and phagocytic cells as well as the general inflammatory response, is less energetically-costly to develop, but more costly to activate than acquired immunity, which involves B and T-lymphocytes that use highly specialized antigens to recognize and target pathogens for destruction (Thomas W. McDade, Georgiev, & Kuzawa, 2016). Males with surplus energetic reserves during childhood may be better able to develop acquired immune function, freeing up energy from costly innate immune activation for the expression of high T and T-associated traits. These kinds of tradeoffs within the immune system could obscure tradeoffs between T and immune function in men. Although both innate and acquired measures of immunity have been studied in relation to T (Nowak et al., 2018; Prall & Muehlenbein, 2015; Trumble et al., 2016), they are typically based on cytokine measures alone, and tradeoffs between the two axes of immunity are rarely explored. Other measures, such as leukocyte cell composition could provide additional insights into early developmental tradeoffs within the immune system by explicitly testing for tradeoffs along an axis of acquired vs. innate immunity.

A new approach that holds great promise for studying T-associated CoR in men involves the study of epigenetic processes. Epigenetic processes are a set of biochemical and cellular metabolic processes that are involved in chromatin packaging, gene regulation, and cellular memory. One epigenetic process in particular, DNA methylation (DNAm), provides researchers with a range of tools for studying T-

associated tradeoffs in men. DNAm involves the covalent attachment of a methyl group to DNA, often linked to stable changes in gene regulation in nearby genes. Changes in DNAm accompany development and aging and can be used to predict disease and mortality (McEwen, Goodman, Kobor, & Jones, 2017; D. S. Moore, 2015; T. Tollefsbol, 2012). Specifically, DNAm can be used for 'epigenetic clocks', which predict health and mortality with greater accuracy than other biomarkers of aging or chronological age alone (Horvath, 2013; Levine et al., 2018; Lu, Quach, et al., 2019; Ryan, 2020). These clocks allow researchers to predict mortality risk even in relatively young and apparently healthy subjects, and even in the absence of other biomarkers. DNAm can also be used to bioinformatically-derive immune cell composition, providing a means of testing immunomodulatory effect of T which could have long-term impacts on inflammation, aging, and cancer. Finally, exploratory genome-wide scans of DNAm as they relate to T could provide insights into gene regulation more broadly, which could lead to novel hypotheses when theorized costs may not be empirically-supported (Biesecker, 2013).

Here, we aim to clarify the role of T-induced CoR by studying the relationship between T and measures of men's health, immunity, and mortality. We capitalize on three measures of T and DNAm in a sample of 90 young (20-22) men in Cebu City, Philippines (Adair et al., 2011; Kuzawa et al., 2020). In order to account for costs that may be confounded by social or nutritional conditions, we control for smoking status, socioeconomic status, body mass index, and genetic variation. The three measures of T – waking salivary T, pre-bed salivary T, and daytime total plasma T – reflect distinct biological fractions of T that are thought to capture different aspects of testosterone's

regulation and biological activity. We use DNAm to estimate epigenetic age using four distinct epigenetic clocks (Hannum et al., 2013; Horvath, 2013; Levine et al., 2018; Lu, Quach, et al., 2019). These clocks provide insights into separate but overlapping aspects of biological aging, metabolic and inflammation, and mortality risk (Ryan, 2020). We also use bioinformatically-derived estimates of leukocyte proportions for CD4T cells, CD8T cells, plasma blastocysts, natural killer cells, monocytes, and granulocytes (Houseman et al., 2012a). To test for tradeoffs within the immune system, we also collapse cell types along an axis of innate-acquired immunity, and examine whether these axes are associated with all three T measures. Finally, we take a more agnostic approach to the potential pathways involved in T-associated CoR in men by employing an epigenome-wide association for DNAm at 144,777 locations across the genome. This allows us to take a hypothesis-generating approach to the molecular effects of T on men's health.

Table 5-1. Findings summarizing relationship between testosterone (T), predicted public health patterns in men, and current evidence in humans. Putative pathways, which might provide a mechanistic link between theory and biology, are highlighted, with those investigated in the current study underlined.

	Associated with T	**Predicted public health outcomes**	**Evidence in humans**	**Predicted Pathways**
Risky behavior	Yes. T both regulates and responds to T.	Men with high T expected to die from accidents, violence, suicide.	Single and childless men have higher T and die more often from accidents, alcohol, drug-related death, suicide.	Neurological, HPA-axis, dopamine, norepinephrine
Metabolism	Yes. Higher T associated with lean muscle mass and metabolism.	Men with high T expected to die of famine faster than women.	Men tend to die sooner during periods of famine than women.	Glycolysis, lipolysis, oxidative stress
Immunocompetence	Mixed. Mostly in vitro.	Men expected to be more prone to bacterial and viral (e.g. Sars-CoV-2) infection.	Mixed evidence. Positive 'phenotypic correlations', but also monocyte count increase.	Immunological. Changes in cell complement system or cell composition.

Methods

Sample and data collection

Data come from participants in the Cebu Longitudinal Health and Nutritional Survey (CLHNS), a community birth cohort study in Metropolitan Cebu, Philippines that began with enrollment of 3,327 pregnant mothers in 1983–1984 and is ongoing to the present (Adair et al., 2011; Kuzawa et al., 2020). These analyses examined 90 men (20-22 years old at the time of the study) for whom all necessary 2005 questionnaire data, testosterone data, genetic information, and DNA methylation data were available. Surveys and anthropometrics were conducted during in-home visit by trained, Cebuano-speaking research staff. Details on biological sample collection are provided in more detail below. This research was conducted under conditions of written informed consent with human subjects clearance from the Institutional Review Boards of Northwestern University and the University of Chapel Hill, North Carolina. Survey data are available for download at: https://dataverse.unc.edu/dataverse/cebu.

Testosterone measurement

To account for different fractions (bioavailable vs. total T) and circadian effects on T, three measures of T were used: waking salivary testosterone (AM-T), salivary testosterone before bed (PM-T), and total plasma T. Saliva and plasma samples were generally taken during the same 24-hour period, although 6 of 90 men had salivary samples taken more than 2 days apart from blood samples. For plasma T, participants

were asked to fast overnight for 12 hours, and blood samples were taken the following morning using EDTA-coated tubes. Mean time of blood draw was 07:07 (range 05:40–09:30). After separation, samples were frozen and shipped on dry ice to Northwestern University for analysis. Plasma T was analyzed with a commercially available enzyme immunoassay (Diagnostic Systems Laboratories #DSL-10-4000, Webster, TX). All samples were assayed in duplicate, and control samples were included with each assay to monitor between-assay variation. The coefficients of variation for low and high controls were, 13.3% and 5.8%, respectively. Because the time of day affects testosterone levels, we used the residuals corrected for time of sample collection for all analyses.

For salivary T (AM-T and PM-T), each participant was provided with instructions and two tubes for saliva collection. The first sample was collected immediately prior to bed (PM-T). After collection, tubes were sealed and kept at room temperature. Participants were instructed to place the second tube next to their bed and to collect the second sample immediately upon waking the following morning (AM-T). At each collection time, the participant was asked to record the time of collection, with average PM-T and AM-T collection times being 22:23 and 06:34, respectively. Tubes were collected later that day, and immediately placed on ice packs in a cooler by an interviewer. Tubes were then transported to a freezer where they were stored at -35°C until shipment on dry ice to the Laboratory for Human Biology at Northwestern University, where they were stored at -80°C. Samples were thawed, centrifuged, supernatant separated, and aliquoted into smaller tubes for subsequent analysis of individual analytes. Salivary T

concentrations were determined in duplicate using an enzyme immunoassay protocol developed and validated for use with saliva samples (Salimetrics #1-2402, State College, PA). The between-assay coefficients of variation were 5.6% and 6.7% for high and low controls, respectively. Because the time of day affects testosterone levels, we used the residuals corrected for time of sample collection for all analyses.

DNA methylation processing

DNAm was derived from the same 2005 blood samples used for plasma T. Blood samples from overnight fasted subjects were collected by venipuncture into EDTA-coated vacutainer tubes. Automated and manual DNA extraction (Puregene, Gentra) was conducted on blood samples. 160ng of sodium bisulfite converted DNA (Zymo AZDNA methylation kit, Zymo Research, Irvine, CA, USA) was applied to the Illumina HumanMethylation450 Bead Chip using manufacturer's standard conditions. Standard methods for background subtraction and color correction were carried out using default parameters in Illumina Genome Studio and exported into R for further analyses. Quality control involved first confirming participant sex and replicate status. Probes associated with known single nucleotide polymorphisms (SNPs), unreliable probes with a detection p value above 0.01, probes with fewer than three beads contributing to signal, and those previously shown to bind to multiple genomic regions were removed. Sex chromosome-associated probes were retained due to their potential involvement in genomic effects of testosterone. For epigenome-wide association, data were quantile normalized (Du et al., 2008) and probe types were normalized using SWAN (Maksimovic et al., 2012). Plate, row, and chip batch effects were assessed using PCA

and corrected using COMBAT (Leek et al., 2012)(sva). Spearman correlations for samples run in duplicate (n = 1) and quadruplicate (n = 4) were high (>0.99), confirming batch effect corrections. Proportions of blood cell types were estimated using previously published algorithm and removed from the main DNAm data using a linear regression approach (Houseman et al., 2012a; Jones, Islam, et al., 2015).

Epigenetic clocks and cell type proportions

For epigenetic clock analyses, quality control of raw DNA methylation was modified as per (Ryan, 2020) as follows: In order to maximize the number of sites available for the epigenetic age calculator, probes with detection p-values above 0.01 were called NA for poor performing samples only, and were otherwise retained. Both XY-binding and SNP-binding probes were retained. Processing involved quantile but not SWAN normalization. Four clocks were examined: Horvath's DNAmAge, Hannum's DNAmAge, Levine's PhenoAge, and Lu's GrimAge. Epigenetic ages for all clocks were calculated using an online calculator (http://labs.genetics.ucla.edu/horvath/dnamage/). Background-corrected beta values were pre-processed using the calculator's internal normalization algorithms. In all models, epigenetic clock values themselves were used as the outcome rather than 'AgeAccel', with age included as a covariate in the model. Cell proportions were also estimated using the online calculator, based on previously described methods (Horvath, 2013; Houseman, Molitor, & Marsit, 2014) Cell types examined as part of the present study included CD4T cells, CD8T cells, plasma blastocysts, natural killer cells, monocytes, and granulocytes.

Survey and anthropometrics measurement

Socioeconomic status, smoking, and body mass index have been associated with DNAm and epigenetic clocks and were included in all models (Beach et al., 2015; Demerath et al., 2015; Huang et al., 2019; Thomas W. McDade et al., 2019; Simons et al., 2016). Using survey data, SES was operationalized as a combination of income, education, and assets. Participants reported their annual income from all sources, including in-kind services, and the sale of livestock or other products by household members during the prior year, which were summed to determine total house-hold income. Incomes from 2005 were deflated to 1983 levels, and log-transformed. Maternal education (in years) was also reported. Participants also reported on nine assets (coded 0, 1) that were selected to capture population-relevant aspects of social class, including electricity, televisions, refrigerators, air conditioners, tape recorder, electric fans, jeepneys, cars, and their residence. In addition, house construction type (i.e., light, mixed, permanent structure) was coded as 0, 1, and 2, respectively. Thus, asset scores ranged from 0 to 11. A principal components analysis was run on log income and assets at birth (1983) and at sample collection (2005) along with maternal education in Stata (v. 14.1). The first component of variation accounted for 49% of the variation and individual scores for this component of variation were used as our measure of SES. Anthropometrics included body weight (kg) and height (cm). The body mass index (BMI) was calculated as the ratio of weight (kg)/height (m^2). Smoking was dichotomized variables (0 = non-smoker/drinker, 1 = occasional or frequent smoker) generated from reports at the time the blood sample was taken.

Statistical analyses

A total of 440,810, probes passed quality control. Many DNAm sites are invariable between individuals and hence unlikely to be informative with respect to individual differences in other exposures or traits (Mill & Heijmans, 2013; Rakyan et al., 2011), in our case salivary or plasma T. We therefore sought to reduce the burden of multiple comparison correction by concentrating our analyses on a subset of probes for which variability in β-values between the 10th and 90th percentiles in these men was >5%. This left us with a subset of 142,777 probes for our epigenome wide association (EWA) scan. Prior to analysis, these were converted from β-values to M-values prior to statistical analyses (Du et al., 2010). For our epigenome-wide association, probe wise variance was determined by fitting linear regression models and applying parametric empirical Bayes smoothing formula over the entire array dataset that passed quality control using the R bioconductor package limma (Ritchie et al., 2015). This approach allowed for gene-wise information borrowing to better estimate the variation for each probe. The model outcomes from the subset of variable probes described above were then extracted, and corrected for multiple comparisons using the method of Benjamini and Hochberg (1995). Models included residuals of each measure of testosterone corrected for time of sample collection, as well as age at the time of blood sample, smoking, BMI, and our composite score of SES. To account for genetic variation, we also included the top-2 principal components of genome-wide SNP variation derived from the Global Screening Array (Infinium Global Screening Array-24 v2.0—Illumina). These were obtained after standard SNP quality control measures, by performing

multidimensional scaling using Euclidean distance to collapse genetic variation into the top-2 most informative dimensions. Similar models were used for epigenetic clock analyses.

Results

Sample characteristics

From an initial sample of 99 men, six men were missing either AM-T, PM-T or time of saliva collection, and were excluded from the analyses. Two men with plasma T levels >3 standard deviations above the mean (65.43 and 136.19 ng/mL) were also excluded. One man was missing genetic data, and was excluded, leaving a total of 90 men for all analyses. A summary of characteristics for the 90 remaining men is in Table 5-2.

Table 5-2. Summary of characteristics of study sample examining potential testosterone (T)-induced costs of reproduction (CoR) in young men (n=90).

Statistic	Mean	St. Dev.	Min	Max
Age (years)	21.74	0.44	21	22
Salivary AM-T (pg/mL)	191.10	73.95	65.52	357.54
Salivary PM-T (pg/mL)	116.45	49.49	40.70	260.74
Plasma T (ng/mL)	7.90	2.67	4.03	17.19
Smoke? (Yes = 1)	0.47	0.50	0	1
Drink? (Yes = 1)	0.83	0.37	0	1
Body Mass Index (kg/m^2)	21.09	2.54	16.27	29.90
SES PC-score	0.06	1.48	-2.73	5.18
Genetic PC-score 1	-1.57	9.60	-19.05	21.52
Genetic PC-score 2	-0.77	9.10	-16.42	35.20

*Residuals of AM-T, PM-T, and plasma T from regression on time of saliva or blood sample used for all analyses
**All analyses used logged plasma T to account for right skew in the data

Epigenetic clocks and cell types

To assess the effect of testosterone on aging and mortality risk, we looked at the relationship between AM-T, PM-T, and plasma-T and four epigenetic clocks: Horvath's DNAmAge, Hannum's DNAmAge, Levine's DNAmPhenoAge, and Lu's DNAmGrimAge. All clocks were positively associated with AM-T, but this relationship was only significant for Levine's DNAmPhenoAge. This effect remained when correcting for false discovery rate from examining four different clocks (false discovery q-value = 0.036). All clocks were also positively associated with PM-T and plasma T, but these relationships were not statistically significant for any of the epigenetic clocks (Fig. 5-1; Table 5-3).

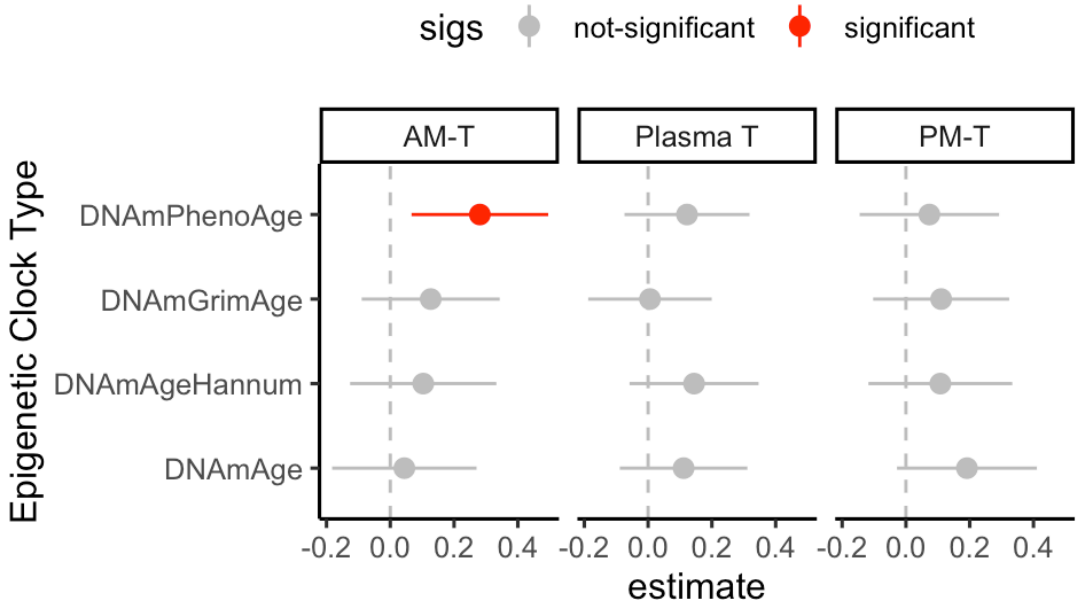

Figure 5-1. Forest plot for regression models examining the relationship between different measures of testosterone (AM-T, PM-T, and plasma-T) and epigenetic age acceleration for four epigenetic clocks (Horvath's DNAmAge, Hannum's DNAmAge, Levine's DNAmPhenoAge, and Lu's DNAmGrimAge). Significant associations between testosterone and epigenetic clocks are highlighted in red. All testosterone measurements corrected for time of sample, and all models included smoking status, body mass index, multidimensional scaling of the top 2 principle components of genetic

variation, and a principle component based on socioeconomic status in the year the man was born and the year the sample was taken.

Table 5-3. Table of regression effects and p-values for full models examining relationships between three different testosterone measurements and four epigenetic clocks (Horvath's DNAmAge, Hannum's DNAmAge, Levine's DNAmPhenoAge, and Lu's DNAmGrimAge). Statistically significant relationships are denoted by an asterisk (see notes below).

	Horvath (1)	**Hannum** (2)	**Pheno** (3)	**Grim** (4)	**Horvath** (5)	**Hannum** (6)	**Pheno** (7)	**Grim** (8)	**Horvath** (9)	**Hannum** (10)	**Pheno** (11)	**Grim** (12)
AM-T	0.040	0.098	0.279	0.128								
	p = 0.730	p = 0.396	p = 0.013*	p = 0.253								
PM-T					0.195	0.113	0.075	0.110				
					p = 0.081+	p = 0.323	p = 0.505	p = 0.319				
Plasma-T									0.142	0.180	0.135	0.0005
									p = 0.166	p = 0.081+	p = 0.186	p = 0.997
Age	0.183	0.212	0.062	-0.039	0.188	0.216	0.069	-0.034	0.207	0.244	0.090	-0.036
	p = 0.090+	p = 0.052+	p = 0.549	p = 0.710	p = 0.077+	p = 0.047*	p = 0.517	p = 0.745	p = 0.056+	p = 0.026*	p = 0.402	p = 0.735
Smoke = Y	0.079	-0.102	-0.051	0.600	0.089	-0.128	-0.160	0.561	0.046	-0.164	-0.185	0.546
	p = 0.716	p = 0.641	p = 0.806	p = 0.006**	p = 0.670	p = 0.549	p = 0.450	p = 0.008**	p = 0.827	p = 0.436	p = 0.377	p = 0.010**
SES	-0.068	-0.224	-0.298	-0.303	-0.069	-0.211	-0.253	-0.285	-0.060	-0.205	-0.248	-0.281
	p = 0.541	p = 0.049*	p = 0.007**	p = 0.007**	p = 0.525	p = 0.059+	p = 0.023*	p = 0.009**	p = 0.581	p = 0.063+	p = 0.024*	p = 0.011*
BMI	-0.263	-0.046	-0.238	0.004	-0.241	-0.014	-0.160	0.043	-0.267	-0.039	-0.178	0.037
	p = 0.022*	p = 0.682	p = 0.031*	p = 0.975	p = 0.027*	p = 0.896	p = 0.141	p = 0.683	p = 0.015*	p = 0.718	p = 0.101	p = 0.730

Genetic PC-Score 1	-0.136	0.020	0.088	-0.043	-0.136	0.028	0.117	-0.032	-0.124	0.041	0.127	-0.029
	p = 0.200	p = 0.854	p = 0.389	p = 0.675	p = 0.190	p = 0.791	p = 0.267	p = 0.758	p = 0.238	p = 0.693	p = 0.226	p = 0.777
Genetic PC-Score 2	0.014	0.028	-0.163	-0.104	0.056	0.051	-0.153	-0.082	0.024	0.040	-0.159	-0.107
	p = 0.899	p = 0.796	p = 0.117	p = 0.320	p = 0.602	p = 0.648	p = 0.164	p = 0.442	p = 0.822	p = 0.710	p = 0.136	p = 0.311
Intercept	-0.027	0.069	0.032	-0.272	-0.021	0.087	0.085	-0.248	-0.013	0.096	0.092	-0.247
	p = 0.851	p = 0.635	p = 0.818	p = 0.055^{+}	p = 0.879	p = 0.545	p = 0.548	p = 0.077^{+}	p = 0.927	p = 0.497	p = 0.513	p = 0.080^{+}
Observations	90	90	90	90	90	90	90	90	90	90	90	90
Adjusted R^2	0.062	0.029	0.134	0.132	0.096	0.033	0.071	0.128	0.083	0.057	0. 085	0.118

Note: +p<0.1;*$p<0.05$;**$p<0.01$;***p<0.001

To test for an immunosuppressive effect of T, we looked at the relationships between AM-T, PM-T, plasma-T and bioinformatically-derived white blood cell count (plasma blastocysts, natural killer cells, monocytes, granulocytes, CD8T cells and CD4T cells). None of the testosterone measures were related to any cell types, before or after correcting for false discovery across all cell types (Fig. 5-2).

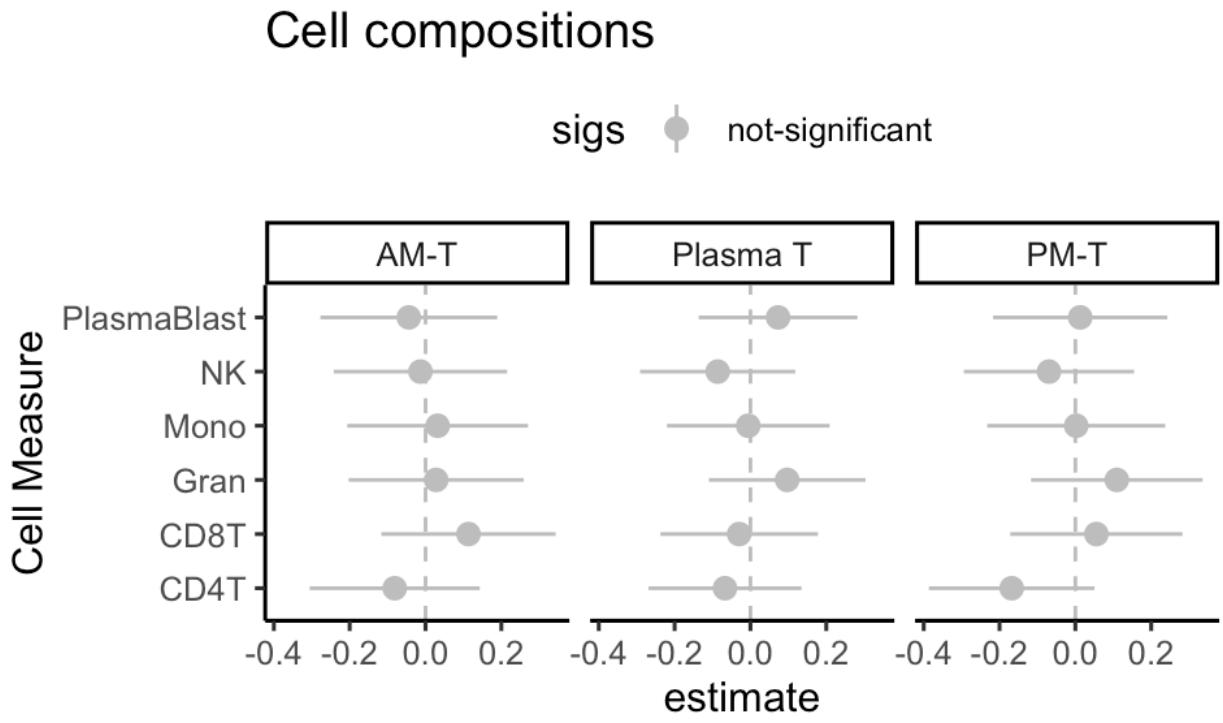

Figure 5-2. Forest plot for regression models examining the relationship between different measures of testostserone (AM-T, PM-T, and plasma-T) and bioinformatically-derived leukocyte cell count proportions.

To test for an effect of T on innate vs. acquired immunity, we first used principle component analysis to collapse blood cell counts into fewer dimensions. The first 3 principle components explained 70.9% of the variance in white blood cell count, with the first dimension (variance explained = 45.9%) lining up along an axis consistent with innate/acquired immunity (Fig. 5-3). The second and third dimensions largely partitioned

out the variance associated with being natural killer cells and monocytes, respectively (Fig. 5-3). However, none of AM-T, PM-T, and plasma T were associated with any of the first 3 dimensions of cell proportions, even before correcting for false discovery rate.

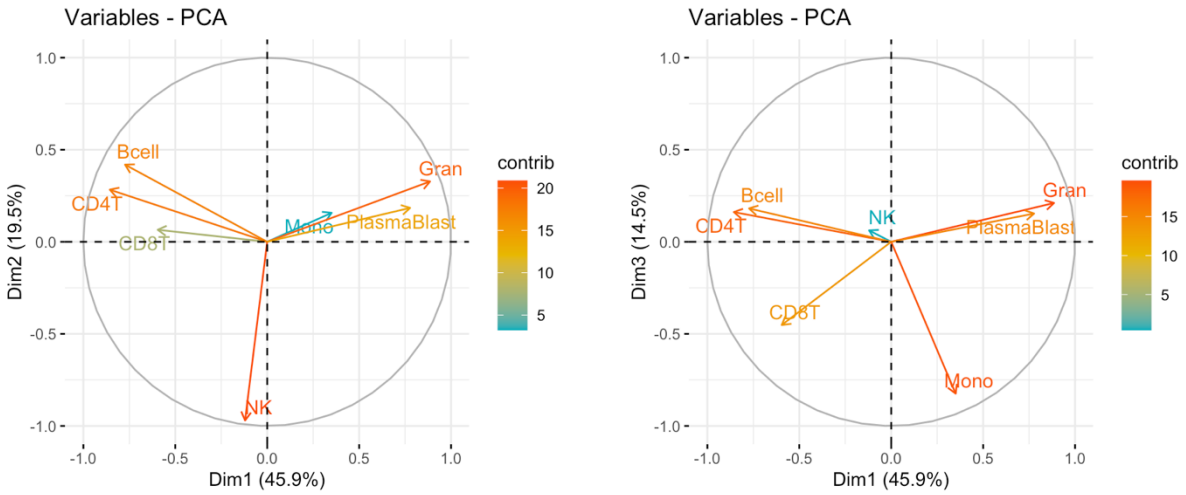

Figure 5-3. Loading plot showing influence of bioinformatically-derived cell type proportion on the top 3 principle components and the relationship between the PC-1 and PC-2, and PC-1 and PC-3. (NK = natural killer cells, Mono = monocytes, Gran = granulocytes, PlasmaBlast = plasma blastocysts).

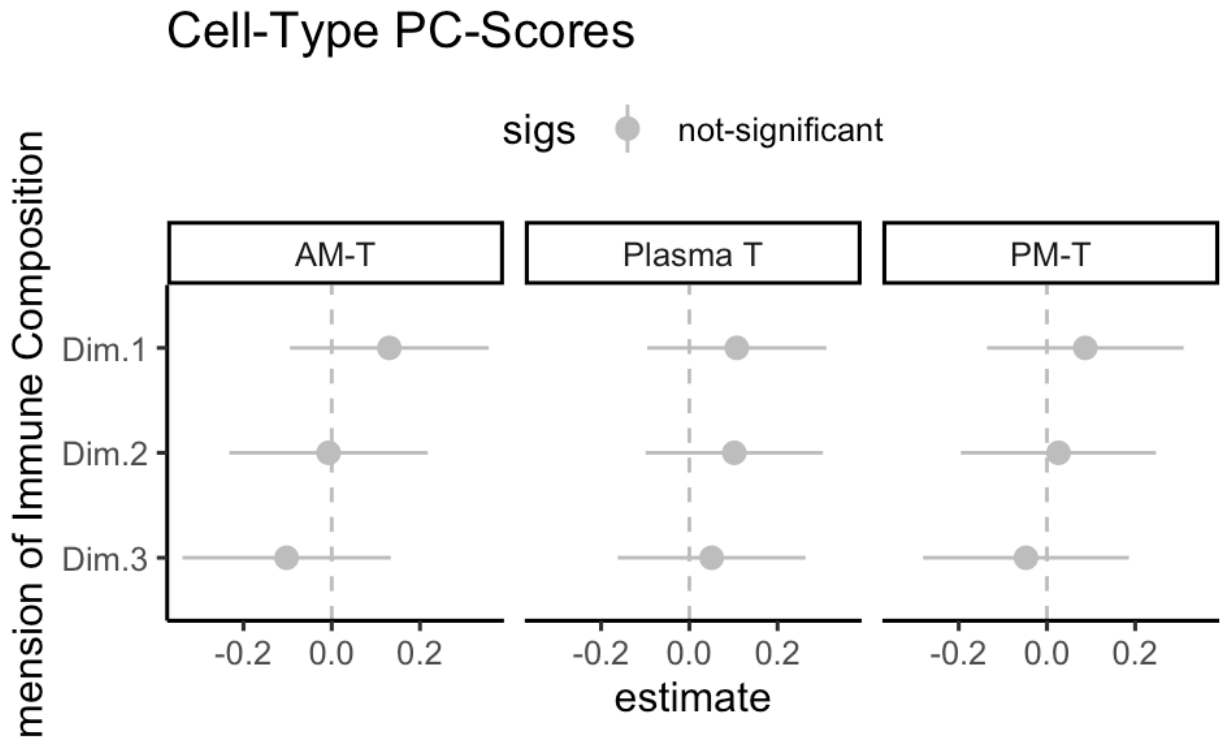

Figure 5-4. Forest plot for regression models examining relationships between different measures of testostserone (AM-T, PM-T, and plasma-T) and top 3 principle components of bioinformatically-derived blood cell proportion. Dimension 1 provides a strong proxy for innate vs. acquired immunity, with positive values indicating greater tendency towards innate immunity.

Epigenome-wide associations with testosterone

To explore potentially novel associations between testosterone and men's health, we evaluated the relationships between AM-T, PM-T and plasma T and DNA methylation at 142,777 CpG sites in the genome. We conducted sensitivity analyses that included testosterone and age only; testosterone, age, smoking status and body mass index (BMI); testosterone, age, smoking status, BMI and socioeconomic status (SES); testosterone, age, smoking status and BMI, SES, and the top 2 principle components of genetic variation based on multidimensional scaling. We did not find any significant

associations between DNAm at any of the sites we examined and AM-T, PM-T or plasma T after correcting for false discovery for any of the tests described. One site associated with PM-T approached statistical significance (FDR q-value = 0.12). This site was associated with the RNF128 (Ring Finger Protein 128) gene, which codes for a transmembrane protein involved in endocytosis. Expression of this gene inhibits IL2 and IL4 cytokine production, and is thought to be involved in an anergic phenotype in CD4(+) T cells (Maglott, Ostell, Pruitt, & Tatusova, 2007). Two other sites for PM-T were under FDR q-value 0.2, associated with genes PPP1R12C and AK056252. PPP1R12C mutants are reported to have altered growth and body size (Bult et al., 2019), as well as altered response to vaccine in cell culture experiments (Schmidt et al., 2013). AK056252 is associated with a poorly characterized long non-coding RNA (lncRNA)(Stelzer et al., 2016). For all models, p-values mostly followed a uniform distribution as would be expected under the null model. However, quantile-quantile plots of p-values from AM-T and plasma T suggested slight evidence for deflated p-values, indicating that p-values closer to 0 were less common than expected by chance. Examples of these distribution from the full model are provided in Fig. 5-5.

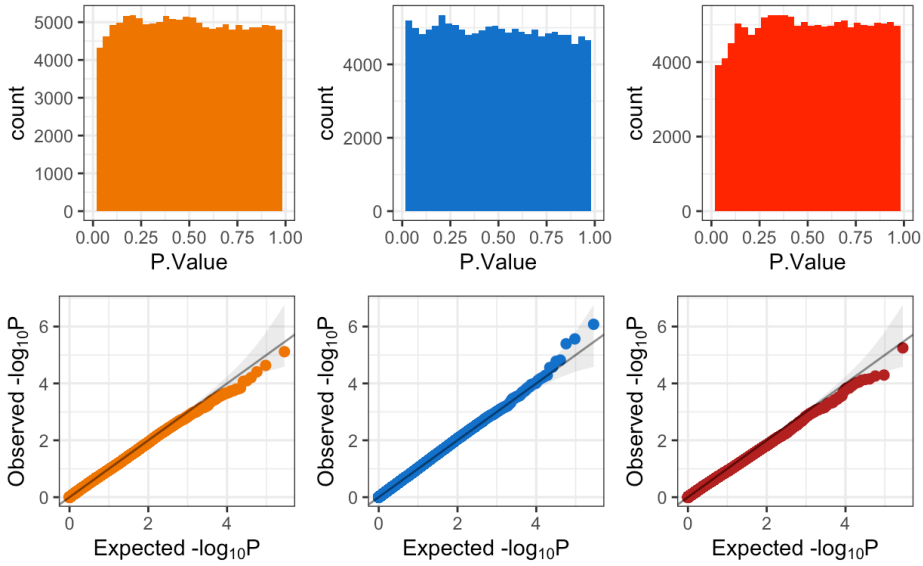

Figure 5-5. Histograms (top) and Quantile-Quantile (QQ) plots (bottom) of observed versus expected p-values (AM-T, left and orange, PM-T, middle and blue, plasma T, right and red). P-value distributions and QQ plots for AM-T and plasma T suggest slightly deflated p-values (fewer than expected p-values close to 0).

Discussion

Testosterone (T) is widely-hypothesized to be a source for costs of reproduction (CoR) in men, which are thought to arise through the hormone's behavioral, metabolic, or immunosuppressive effects. Some T-induced CoR may be evident in changes in the regulation in gene expression, molecular aging, or changes in cell composition, all of which could involve DNA methylation (DNAm) and other epigenetic processes. We used three measures of T (AM-T, PM-T, and plasma T) along with DNAm to test possible T-associated CoR in a cohort of 90 young men (20-22 years) in the Philippines. We first used genome-wide DNAm to calculate epigenetic age for each man using four different epigenetic clocks. These measures reflect related but distinct components of immunological, metabolic, and cellular aging. We then used bioinformatically-derived

blood cell counts to test for specific impacts of T on cell composition. We collapsed blood cell composition into principle components which reflect an axis of innate-acquired immunity. Finally, we took a more agnostic, hypothesis generating approach by looking at the relationship between measures of T and DNAm across 142,777 sites.

Testosterone and Epigenetics clocks

All four epigenetic clocks were positively associated with all three measures of T. This relationship was only statistically significant between AM-T and Levine's DNAmPhenoAge, although PM-T and Horvath's epigenetic age and plasma T and Hannum's epigenetic age were also borderline significant. Levine's DNAmPhenoAge clock is based on a measure trained using 9 clinically-based biomarkers to predict mortality (Levine et al., 2018). The markers used to design Levine's clock are associated with functional integrity of the liver (albumin, alkaline phosphatase) and kidney (creatinine), as well as metabolism (blood glucose levels), inflammation (C-reactive protein), and immune function (lymphocyte percent, red cell volume, red cell distribution width, white blood cell count). This measure forms the basis of DNAmPhenoAge, and also predicts cancer, cardiovascular disease, diabetes, Alzheimer's, and chronic lower respiratory disease (Levine et al., 2018). Our findings provide modest support for the idea that elevated T comes with a 'cost' to health and lifespan, possibly through effects on metabolism, inflammation, or immune function.

It is not clear why DNAmPhenoAge alone would show this relationship. One reason could be the age of samples used to train the clocks themselves. Lu's DNAmGrimAge is

a stronger predictor of all-cause mortality than Levine's DNAmPhenoAge clock, but was trained and validated in datasets comprised predominantly of older (55+ years) individuals (Lu, Quach, et al., 2019). In contrast, Levine's clock was based on NHANES data for individuals 20 years and older, which may perform better in our population of young 20-22 year old men (Levine et al., 2018). Horvath's clock included children and adolescents, and Hannum's clock was trained in a dataset with individuals as young as 19 years of age. However, these clocks were trained to predict chronological age, not mortality, and the sample sizes at younger ages were much smaller for both Horvath and Hannum clocks (Hannum et al., 2013; Horvath, 2013).

DNAmPhenoAge was positively associated with AM-T, but not PM-T or plasma T. This may have to do with the fact that these different measures of T reflect distinct physiological fractions. Testosterone in the blood exists in free (unbound) and bound fractions (Norman & Litwack, 1997). The bound fraction of T is associated with either low-affinity albumin, or high-affinity sex hormone binding globulin (SHBG). Plasma T provides a measure of total testosterone, which includes bound and unbound fractions, while our salivary measures capture T that is free or bound weakly to albumin, classically thought to be the 'bioavailable' fraction of this hormone (Simoni, Fanelli, Roli, & Pagotto, 2012). If this is correct, it is fitting that only salivary T is associated with DNAmPhenoAge. The finding that salivary AM-T but not PM-T is associated with DNAmPhenoAge is also interesting in light of research suggesting functional differences between these two measures. Testosterone levels are highest during sleep, decrease rapidly upon waking, and decline gradually throughout the remainder of the day

(Kuzawa, Georgiev, McDade, Bechayda, & Gettler, 2016). This is thought to help reduce conflict between the somatic and anabolic roles of testosterone with social priorities in which high T is less desirable, such as during parenting. Accordingly, in a larger sample of men from this cohort, AM-T (but not PM-T) was related to fat free mass, grip strength, and arm muscle area, although this was only observed in men who also reported high levels of physical activity (Lee T. Gettler, Agustin, & Kuzawa, 2010). This suggests that AM-T may better reflect the costs of T as directly related to its anabolic effects on lean muscle mass and strength, which could involve other tradeoffs with metabolism, immune function or inflammation captured by the DNAmPhenoAge clock.

Testosterone and blood immune measures and cell proportions

AM-T, PM-T and plasma T were all positively associated with the first principle component of cell proportions, which aligned well with an axis of acquired vs. innate immunity. Higher T associated with innate over acquired immunity would be consistent with favoring an immune strategy that involves less energy to develop early in life but more energy to activate when needed (Thomas W. McDade, Georgiev, et al., 2016). However, this effect was not statistically significant for any of the three measures. Furthermore, none of the three measures of T predicted the proportion of bioinformatically-derived cell types themselves (plasma blastocysts, natural killer cells, monocytes, granulocytes, CD8T cells or CD4T cells). The null nature of our findings run counter to the ICHH, yet there is now a growing body of evidence suggesting that T is unrelated to many proxies of immune function in human males. In a study of Tsimane

men, Trumble and colleagues did not find any relationship between urinary T and baseline cytokine production, nor between T and ex vivo antigen stimulated B-cell mediated immunity (Trumble et al., 2016). Although this study did find that higher T was linked to down-regulated T-cell biased cytokine production – consistent with our findings here – these associations were relatively weak and only significant for two of 13 cytokines (Trumble et al., 2016). Nevertheless, this sample consisted mostly of older (40+ years) men. In a sample of younger men (19-36.7 years), Nowak et al. did not find any evidence for relationships between androgens (free T, total T, 5α-dihydrotestosterone or dehydroepiandrosterone) and innate (complement activity, lysozyme activity, phagocytic uptake, reactive oxygen species production) or acquired (immunoglobulin A levels, immunoglobulin G levels, T lymphocyte counts, B lymphocyte counts) immunity (Nowak et al., 2018). The only significant association in the study of Nowak et al. was a positive relationship between free plasma T and a response to a flu vaccine (Nowak et al., 2018). This is consistent with similar positive correlations between T and immunity reported elsewhere (Lee T. Gettler et al., 2014; Rantala et al., 2012), supporting the idea that high quality males may be able to maintain both high T and robust immune response. Thus, T may have subtle immunomodulatory – rather than immunosuppressive – effect, or effects that are obscured by phenotypic correlations or that only become apparent later in life.

Testosterone and genome-wide DNA methylation

Despite the well-established molecular and phenotypic effects of T and other androgens, none of the three measures of T in our study were significantly associated

with DNA methylation after false discovery correction. Our lack of findings is largely consistent with the findings of Arathimos et al. (Arathimos, Sharp, Granell, Tilling, & Relton, 2018), who examined the relationship between DNAm and plasma T, SHBG, and bioavailable T in young (7.5 years) and adolescent (16.5 years) boys. Although they did find evidence for one differentially-methylated region during childhood, T was not associated with differentially-methylated regions during adolescence, nor in any individual CpG sites after correction for false discovery (Arathimos et al., 2018). In contrast to our findings and those of Arathimos et al., Moore et al. found that salivary T was associated with 'modules' of time and sex varying co-methylated genes in adolescents (S. R. Moore et al., 2020). However, these findings were for buccal epithelial cells, not blood leukocytes, and their methods differed considerably from those describe here and by Arathimos et al. (S. R. Moore et al., 2020).

Largely null findings in this study and by Arathimos et al. might be explained by the complex genomic and non-genomic effects of androgens, and their interaction with binding proteins, receptors, and regulatory proteins (Bennett, Gardiner, Hooper, Johnson, & Gobe, 2010). Most androgens, including T, exert their transcriptional genomic effects by binding of the androgen receptor (AR) inside the cell. Conventional understanding is that the 'bioavailable' fraction of T does not include T bound to high-affinity sex hormone binding globulin (SHBG), which comprises roughly half of all T (Luetjens & Weinbauer, 2012). However, the "free-hormone hypothesis" has not been universally supported (Luetjens & Weinbauer, 2012). The binding affinity of T for SHBG is higher than albumin, but plasma albumin levels are high enough that binding

capacities are roughly equal (Luetjens & Weinbauer, 2012). Furthermore, SHBG may interact with cell surface receptors in ways that alter the bioactivity of T and other androgens (Simoni et al., 2012). Thus, the measures of T in our study and others may provide only rough estimates of the bioactivity of T in the leucocytes we used to measure DNAm.

Once in the cell, androgens bind to the AR, which has two short tandem repeat motifs in its N-terminal domain. Genetic variation in these repeats has been linked to AR transcriptional activity (Kazemi-Esfarjani, Trifiro, & Pinsky, 1995; Simanainen et al., 2011). Transcriptional sensitivity of the AR to T and other androgens add a layer of complexity when making associations between circulating hormone levels and genetic regulation as reflected by DNAm. To complicate things further, testosterone is only one of several hormones that bind the AR (Luetjens & Weinbauer, 2012). T is often metabolized to DHT by 5α-reductase in the cell, which binds with higher affinity to AR but produces distinct biological responses by binding to a different subset of androgen-response elements (Luetjens & Weinbauer, 2012). Other steroid hormones, including androstenedione, estradiol, and progesterone also bind to the AR (Luetjens & Weinbauer, 2012). Metabolism to DHT or other hormones therefore depends on the activity of 5α-reductase or other enzymes, whose activity and density can vary by tissue and over time (Simoni et al., 2012). Thus, differences in T metabolism or the effect of other hormones could add unmeasured variation into the relationship between T and DNAm.

In addition to these genomic pathways, T may also exert its biological effects through non-genomic pathways by way of interactions with dozens of other cytoplasmic or membrane bound regulatory proteins (Bennett et al., 2010; Luetjens & Weinbauer, 2012). These interactions complicate a one-to-one relationship between testosterone and gene regulation and DNAm, but could still have important phenotypic effects. Indeed, T-cells, which make up a large portion of the cells used for DNAm in our study do not appear to respond to T through genomic pathways (Luetjens & Weinbauer, 2012), suggesting that effects on these cells must be non-genomic (Henze, Schwinge, & Schramm, 2020). This does not preclude an immunoregulatory effect of T or other androgens but could make detecting such relationships using DNAm more difficult.

Study strengths and limitations

To our knowledge, this is the first study to examine the relationship between testosterone and epigenetic clocks and genome-wide DNAm in adult men. This is somewhat surprising given how commonly T is measured in clinical panels that are often also used to study the DNAm. It is possible that similar studies have been conducted, but also found no statistically significant relationships between T and DNAm and remain unpublished. Epigenetic clocks provide accurate estimates of health and mortality risk, while DNAm offers a window into potentially long-term impacts of T on gene regulation and phenotypic variation. Our bioinformatically-derived estimates of leukocyte composition also provide us with an affordable measure of immune function and health that may be more stable than more widely fluctuating measures, such as secreted immunoglobulins or cytokines. Our study is strengthened by the large

intrasample differences and our ability to statistically control for in factors such as socioeconomic position, smoking status, and body mass index, known to affect DNAm (Beach et al., 2015; Demerath et al., 2015; Huang et al., 2019; Thomas W. McDade et al., 2019; Simons et al., 2016). Furthermore, the fact that we have examined these relationships in young, rather than older men means that our findings are less likely an effect of reverse causation, where older men in poorer health have experienced declines in T.

Nevertheless, this study does have several limitations. Given the variation in testosterone and other factors that affect epigenetic clocks, cell proportions, and DNAm, our sample size of 90 men is relatively small. This constrains our ability to detect statistically significant relationships, particularly as we correct for testing four different clocks, and across 142,777 CpG sites. For example, a sample size of 500 would have detected significant associations 80% of the time for 6 of the 12 clock analyses we conducted, where we only found significant association for DNAmPhenoAge. We also lacked a measure of men's health at the time the samples were taken. Although genome-wide DNAm values and epigenetic clocks are corrected for cell proportions and should be robust to such short-term variation in health, estimates of cell proportions themselves as well as our dimensions of innate vs. acquired immunity could be affected by health status at the time samples were taken. It has also been shown that T levels often decline when men are sick (Michael P. Muehlenbein & Bribiescas, 2005), adding another confounder with the lack of this health information. Another potential issue is that 6 of 90 men had salivary T measured 2 or more days before or after from the blood

sample used for DNAm. While the proportion of men in this range is relatively small, this could affect our ability to detect relationship given that factors such as stress, sleep, and health affect T.

Conclusions

We find modest evidence that waking salivary T is associated with acceleration of an epigenetic clock that predicts morbidity and mortality even in young men. All measures of epigenetic age were positively associated with all measures of T, although this effect was only statistically significant for Levine's DNAmPhenoAge. Larger sample sizes will provide greater statistical power to detect these effects and will allow researchers to include additional sociobehavioral parameters such as partnership and fatherhood status when studying the impact of T on men's health and mortality risk. Specifically, our findings do not support an immunosuppressive effect of T, although there was a non-significant trend towards an immunomodulatory role of T contributing to innate over acquired immunity for all three measures of T. Largely consistent with other epigenome-wide association studies of T, we failed to find evidence that DNAm at individual CpG sites was associated with any measure of T, possibly reflecting the physiological and molecular complexity of androgenic effects in men. Additional studies, with larger sample sizes and more detailed information on men's health will, be important to resolve the extent to which T does – or does not – associate with CoR in men.

Chapter 6. Conclusion

Research framework and synopsis

Life history tradeoffs are fundamental concepts in evolutionary biology (S. C. Stearns, 1989), biological anthropology (Stinson, Bogin, & O'Rourke, 2012), and evolutionary medicine (Grunspan et al., 2018). Among the most well-theorized and commonly studied life history tradeoffs are those occurring between reproduction and somatic maintenance and aging (Sear, 2020). However, tradeoffs between reproduction and somatic maintenance have been difficult to demonstrate definitively in humans (e.g. Gagnon et al., 2009; Samuli Helle, 2019; Hurt, Ronsmans, & Thomas, 2006; Le Bourg, 2007). This is in part because phenotypic correlations and environmental factors complicate the search for simple one-to-one negative relationships, and experimental approaches are unethical and unfeasible (Bolund, 2020; David Reznick et al., 2000). The challenge of studying tradeoffs between reproduction and aging is made more difficult by a lack of concrete understanding of the mechanisms through which such tradeoffs could occur (Flatt & Heyland, 2011; Harshman & Zera, 2007). Many life history traits have undergone rapid secular changes in modern human populations (Corbett, Courtiol, Lummaa, Moorad, & Stearns, 2018), and often exhibit surprisingly low heritability (T. Price & Schluter, 1991). These observations highlight the key role for plasticity and biological sensitivity in tradeoffs between reproduction and aging. However, plasticity also leaves a gap between the genotype and the phenotype – similar in many ways to that described by Waddington for development during the century of the gene (Waddington, 1942). It is uncontroversial to describe natural

selection as the driving force behind life history traits and tradeoffs (Stephen C. Stearns, Byars, Govindaraju, & Ewbank, 2010), and to expect those tradeoffs to be sensitive to physiological and environmental cues (Kuzawa & Bragg, 2012; Stephen C. Stearns & Koella, 1986). But without concrete mechanistic models through which both genetic and environmental information come together to create tradeoffs, researchers are forced to rely on 'black boxes' or metaphors for how life history traits and tradeoffs actually operate (Flatt & Heyland, 2011). Metaphors limit the ability of scholars to refine hypotheses about life history tradeoffs, or to intervene in the health outcomes that are expected to arise from them. A mechanistic framework for studying life history tradeoffs is therefore an important and necessary goal.

A key component of building this mechanistic framework may involve the study of DNA methylation (DNAm) or other epigenetic processes (Duncan, Gluckman, & Dearden, 2014). DNAm is constructed around the genome, allowing researchers to point to specific genes or biological processes, which gives them a specificity that does not naturally arise from many other physiological measures and biomarkers. DNAm is also highly responsive to the environment inside and outside the body, providing a flexible range of tools for studying reproduction and somatic maintenance. This flexibility means that DNAm can be used to bridge the evolutionary (i.e. genetic) and ecological (i.e. social and physical environment) dimensions of life history traits and tradeoffs, vital for truly understanding how tradeoffs between reproduction and maintenance might work.

In Chapter 2, I introduce a striking example of the flexibility of DNAm as a tool in the form of epigenetic clocks. I discuss how DNAm appears to act as a time-keeper, starting

in the earliest stages of differentiation and faithfully tracking life until old age and mortality (Hoshino et al., 2019; Vaiserman, 2018). I describe how epigenetic clocks are being used with extreme precision to predict chronological age (Q. Zhang et al., 2019) as well as mortality (Levine et al., 2018; Lu, Quach, et al., 2019; Y. Zhang et al., 2017) and a long list of other health outcomes and biomarkers (C. Liu et al., 2018; Lu, Quach, et al., 2019; Lu, Seeboth, et al., 2019). I discuss how epigenetic clocks are providing a concrete set of biomarkers of biological aging and mortality that can be used to gauge life span and mortality years or even decades before clinical or demographic endpoints like chronic disease occur (Ryan, 2020). And I describe how these remarkable characteristics are allowing us to use epigenetic clocks to quantify biological aging and to study tradeoffs like those between reproduction and aging in ways that were once unfeasible.

In Chapter 3, I demonstrate the use of epigenetic clocks to study tradeoffs between reproduction and somatic maintenance by looking at the relationship between gravidity and Horvath's epigenetic age acceleration in 397 young women in Cebu (Ryan et al., 2018). I show that with each additional pregnancy, women's epigenetic age is accelerated by between 4-6 months, even among these relatively young (20-22 years old) women. These findings are corroborated by a similar finding for leukocyte telomere length, a separate marker of cellular aging. In addition, the fact that epigenetic age was unrelated to parity in the four years subsequent to the epigenetic age measurement suggests the relationships we observed are not due to a separate genetic or social confounder that both increases fertility and accelerates epigenetic age (Ryan et al.,

2018). Analyses also account for several measures of socioeconomic status and genetic variation, which help reduce confounding in these analyses. An unexpected finding of this study was the fact that pregnant women looked epigenetically younger according to Horvath's clock (Ryan et al., 2018). The reasons for this relationship are still unclear, but could be related to the large shifts in immune regulation and inflammation that accompany pregnancy (Aghaeepour et al., 2017), or fetal contamination of maternal blood (i.e. fetal microchimerism)(Boddy, Fortunato, Sayres, & Aktipis, 2015).

The paradoxical finding that women appear more epigenetically youthful during pregnancy, despite exhibiting epigenetic age acceleration in relation to parity, could provide a clue as to when tradeoffs between reproduction and aging actually arise, and an opportunity to study the pathways involved. I investigate this possibility in more detail in Chapter 4, where I examined the methylome in relation to reproductive status among the same women described in Chapter 2. By characterizing the differences in the methylome of women who are of roughly the same age, but who vary in current reproductive status, I sought to move beyond the epigenetic clock to the actual regulatory changes that might accompany reproductive effort itself. Compared to nulliparity, there were a large number of differences in the methylomes of pregnant (823 loci) and breastfeeding (1107 loci) women. Interestingly, whereas pregnancy was predominantly associated with hypomethylation, breastfeeding was associated with the opposite trend – hypermethylation. Only a small proportion (8%) of these differences overlapped, highlighting the clear distinction between these two stages of reproductive

investment. Taking these analyses further, I characterized the differentially methylated positions (DMPs) by their genomic context, ranked associated genes using effect sizes and p-values, and described the biological processes associated with top-ranking genes using network analysis. Pregnancy was associated with changes in T-cell regulation and cell-cell adhesion, consistent with immunological adaptations required for fetal tolerance. These could be tied to the activity of T regulatory cells (Tregs), which suppress T cell induction and prevent an immune response to the semi-allogeneic fetus (La Rocca, Carbone, Longobardi, & Matarese, 2014). Tregs also have important roles in cancer and autoimmune disorders, diseases that have both been tied to parity (Förger & Villiger, 2020), and may merit further investigation for their role in costs (and benefits) of reproduction. The other major pattern of DNAm accompanying pregnancy was neuron development and differentiation. This remarkable finding is consistent with neurological and cognitive changes that are becoming more well-recognized both during and after pregnancy (Beeri et al., 2009; de Lange et al., 2019; Hoekzema et al., 2017). While the effects of parity on cognitive function and brain structure in women are not universally detrimental (de Lange et al., 2019), these findings may prove relevant to emerging theories in human biology that view costs of reproduction as involving brain-associated changes and faster age-related cognitive decline (Ziomkiewicz, Wichary, & Jasienska, 2019).

The analyses in Chapter 4 were conducted using cross-sectional data, but we are interested in the long-term effects of reproductive history on women's epigenome and health. To gain some sense for the effects of reproduction on the methylome over time,

I compared nulliparous women and parous (but no longer pregnant or breastfeeding) women. I did not find significant differences in DNA methlylation for any individual loci. However, the non-parametric enrichment analysis (which does not rely on statistically significant differences for individual CpG loci) pointed again to changes in neuron development and axonogenesis (Chapter 4). We also found evidence for persistent differences in immune cell composition between nulliparous and parous women, which are consistent with more durable effects of parity on the methylome. These findings agree with the findings for pregnancy described above, and complement research in other samples showing changes in brain structure and function in parous women (Beeri et al., 2009). Although cross-sectional, these findings represent an important step towards building a mechanistic framework for the costs of reproduction in women using DNAm. Future work using multiple measures of DNAm and longitudinal data from the same women will help us to understand the changes in the methylome that appear accompany women's reproduction, and how those might be related to women's health and aging later in life.

Due to the extensive physiological, immunological, or anatomical changes that women's bodies undergo during pregnancy and breastfeeding, studies of tradeoffs between reproduction and aging often focus on women (Jasienska et al., 2017). Nevertheless, indirect costs of reproduction are expected to exist among men through mating or parenting effort (Bribiescas, 2001; Jasienska et al., 2017). Mating effort in men is thought to be mediated by testosterone (T), which is widely theorized to be costly to male health and longevity (Bribiescas, 2001, 2016). Support for this postulation in

human males, however, has been surprisingly inconsistent (Lee T. Gettler et al., 2014; Michael P. Muehlenbein & Bribiescas, 2005; Nowak et al., 2018; Prall & Muehlenbein, 2015; Trumble et al., 2016). In Chapter 5, I tested this hypothesis by examining the relationship between DNAm and three different measures of T in a subset of 90 young (20-22 year old) men in Cebu. I found evidence that biological age quantified using four different epigenetic clocks was accelerated for all three measures of T. This effect was borderline significant for two clocks (Horvath and Hannum), but only statistically significant for one (Levine's DNAmPhenoAge)(Levine et al., 2018). This suggests that higher T in young men is indeed associated with an increase in biological aging and mortality risk. However, there is little evidence that this effect is tied directly to immunosuppression, since neither bioinformatically-derived immune cell proportions nor a composite measure of innate-acquired immunity was clearly linked to any measure of T. We also found no statistically significant differences in the methylome overall with T, although several sites in genes associated with immune function did approach significance for evening salivary T. These analyses should be viewed as exploratory in light of the relatively small sample of men for whom DNAm were available. However, these preliminary findings, which include consistent directional relationships with multiple epigenetic age markers, including a handful that were borderline significant or significant, along with borderline significant patterns of differential methylation, point to the potential of using DNAm to study T-associated costs of reproduction in men using more well-powered studies.

Future directions in the CLHNS

The premise of this dissertation is that epigenetic measures provide new tools to study life history traits and related tradeoffs. The work presented here has given us a glimpse into how DNAm can be used to study costs of reproduction in both men and women. It also lays the foundation for future research into tradeoffs in epigenome more broadly. As briefly discussed, future studies using longitudinal data, larger sample sizes, and individuals later in life will be an important next step in this research.

While our study on gravidity described in Chapter 2 controls for a range of social, environmental, and genetic factors (Ryan et al., 2018), given the cross-sectional nature of this research we cannot rule out a role for individual differences in health or access to resources that also affect DNAm and epigenetic age. We are addressing this gap by updating reproductive histories and adding a second measure of DNAm as part of a 10-year follow-up. This work is funded by my collaborators (Dr. Chris Kuzawa, Northwestern; Dr. Michael Kobor, University of British-Columbia) and I (through my NSF DDIG) and is well-underway. DNAm has been measured for 335 pregnant women (with DNAm during breastfeeding for 35 of them), and is now ready for signal normalization, probe-type correction, and quality control, which will begin in November, 2020. These data were originally intended to form the final data chapter, but were delayed due to the global coronavirus pandemic. Expanding this work to include a second time point will allow us to use each woman's baseline value as their own control, thereby minimizing confounding tied to individual variation in health and access to resources. These analyses will also produce new data on DNAm at 800,000+ loci per individual, allowing

future investigations of the effect of reproduction and the environment on the methylome. More importantly, our epigenetic data will be complemented by measures of health for women approaching middle age, when the tradeoffs between reproduction and aging may begin to accumulate in the body.

The sample sizes used in the analyses presented here, while large by the standards of most anthropological research, are modest for an epigenomics study that can involve hundreds of thousands of tests. This is especially true for our study of T-associated costs of reproduction in men, for which we had complete data for only 90 men. We are addressing this limitation through the addition of hundreds of DNAm samples for both men and women in 2005 as part of a funded NIH R01 titled ‘Lifecourse determinants and outcomes of epigenetic age acceleration across two generations’. I was instrumental in the conceptualization and writing of this grant, which dovetails with and builds on the work laid out in my dissertation. The larger sample generated by this grant will allow us to retest the hypotheses described above with much improved statistical power. In women, we will have greater power to examine the relationship between parity and DNAm beyond sites associated with epigenetic clocks across the genome, and to study the impacts of pregnancy-associated changes to immune function and DNAm and infant and maternal health. In men, we will be able to study the epigenetic changes that accompany partnership and fatherhood, which was not possible owing to small cell sizes in our present sample. We also plan on analyzing DNAm in the mothers of the original index children, who are now between 56-83 years old. We will combine DNAm measured in whole blood in 2005 when women were 39-66 years old with a

study of health and aging in these women conducted in 2014. We will also update mortality and other outcomes for these women as part of a 2022 survey. These data will allow us to explicitly test the relationships between epigenetic age and health and mortality in these women, which has yet to be done in a non-WEIRD population.

Future directions in anthropology and beyond

My training in life history theory and epigenetic methods are not restricted to questions about reproduction and aging, but extend naturally to other pressing concerns within anthropology and beyond. For example, recent research has found evidence for tradeoffs between growth and immune function in children exposed to high infectious disease burdens (Georgiev, Kuzawa, & McDade, 2016; T.W. McDade, Reyes-García, Tanner, Huanca, & Leonard, 2008). These appear to operate over a range of timescales depending on the nature of the immune response (Urlacher et al., 2018). The flexibility of DNAm could provide us with new ways of studying these tradeoffs. One hypothesis is that intrinsic epigenetic age – thought to capture changes in the pace of biological aging that are not tied to changes in cell composition – will be slowed in children, whereas extrinsic age, specifically capturing immunosenescence, will speed up (Ryan, 2020). This more youthful intrinsic epigenetic age during development may reverse at puberty, with adults exposed to higher infectious disease burdens showing faster pace of intrinsic biological age due to early life tradeoffs between growth and maintenance. A number of other questions could be answered with DNAm: What genome-wide changes in DNAm accompany these tradeoffs? Could DNAm tell us more about what genes

specifically are involved in these tradeoffs? The mechanistic framework and tools provided by DNAm may help us to better understand these processes.

Studies in humans are only one way of understanding the mechanisms of life history traits and tradeoffs. A growing body of research is using free-living primates as systems in which to study the epigenetics of social inequality (e.g. Snyder-Mackler et al., 2016; Tung, Archie, Altmann, & Alberts, 2016) and researchers are recognizing the value of free-living populations for the study of aging (Chiou et al., 2020; Emery Thompson, Rosati, & Snyder-Mackler, 2020). Recently, an epigenetic clock for primate aging has been developed (Goldman et al., 2019), but appears to rely on large-scale bisulfite sequencing, an expensive and labor-intensive method. I am interested in developing a cost-effective method for studying the epigenetic clock using a new technology called ‘high accuracy methylation via Targeted Bisulfite Sequencing’. This development will facilitate research in macaques by providing a low-cost alternative to studying the epigenetic clock in this species. An affordable and widely-applicable epigenetic clock in macaques could be used to study a number of questions about development, reproductive timing and effort, and aging.

Developments in epigenetics are providing us with a powerful set of tools for studying aging, but are also driving a conceptual shift towards viewing aging as an extension of the developmental program set in motion early in life. This ‘lifecourse’ perspective is entirely consistent with ideas about life history strategies common to ecology and evolutionary biology, but often overlooked in biomedicine and gerontology. But the lifecourse perspective of aging is more than a nice idea. Just as developmental

biologists in the 20th century showed that Waddington's ball could be rolled uphill, gerontologists are starting to think about turning the epigenetic clock backwards. There is now evidence from human clinical trials that epigenetic aging – and perhaps biological aging – can be reversed (Fahy et al., 2019). Part of Fahy and colleagues research involved the administration of growth hormone and dehydroepiandrosterone, hormones that are central to growth and reproduction. In this way, studies like that of Fahy et al. can inform research on costs of reproduction or other life history tradeoffs that involve aging. Equally important is the fact that studies of tradeoffs between reproduction and aging in human biology and allied disciplines may provide insights into the genes and pathways involved in certain aspects of biological age, informing clinical research.

In Chapter 2, I provided evidence that women's cells 'age' with each additional pregnancy, supporting the theorized tradeoff between reproduction and bodily maintenance at the cellular level (Ryan et al., 2018). However, despite what appear to be cumulative costs of gravidity on cellular aging, women in our study appeared epigenetically 'younger' during pregnancy itself. What might explain this cellular 'youthfulness' in epigenetic age among pregnant women? One possibility alluded to above is fetal microchimerism – the 'contamination' of the maternal bloodstream by cells from her gestating child. If both maternal and fetal cells contribute to the DNA in a mother's blood sample, our estimates of epigenetic age will be correspondingly skewed downward. To test the potential effect of fetal microchimerism on maternal epigenetic age, Dr. Meaghan Jones at the University of Manitoba and I are carrying out simulations

using several suitable epigenetic datasets to test this hypothesis. We will quantify differences in genetic variation and DNA methylation at Y-chromosome associated genes among mothers of sons versus daughters to validate our findings. If fetal microchimerism contributes significantly to maternal epigenetic age, any study of epigenetic age that includes pregnant women will be biased. If this is correct, our aim will be to provide a statistical algorithm that can be applied in conjunction with existing epigenetic clocks to address this bias in future studies that include pregnant women. It will also provide a novel way of studying the effects of fetal microchimerism using new and existing datasets, which could help us understand how fetal microchimerism may be involved in maternal immune function and health.

While much remains to be learned about how epigenetic processes affect gene expression and phenotypic variation, they are often tied to genes for which we have a greater understanding. For example, the classical research on maternal parenting behavior and its role in epigenetic regulation of the glucocorticoid receptor in the juvenile brain was only meaningful in light of the body of research into the glucocorticoid receptor, its functions in the body and brain, and the phenotypes associated with naturally-occurring or experimentally-induced manipulations of the glucocorticoid receptor gene (Meaney, 2001; Weaver et al., 2004). Follow-up research on childhood abuse in the brains of suicide victims corroborated these findings in humans (McGowan et al., 2009), highlighting the power of comparative approaches. Depending on the gene and research question, insights about human life history evolution can come from any number of model or non-model organisms ranging from yeast and nematodes to mice

and human cell lines (Ashburner et al., 2000). In this way, findings from epigenetic research can build on the foundations of genetics and genomics, providing researchers with conceptual and mechanistic framework for studying context-dependency in life history traits and tradeoffs. By pointing to specific genes, pathways, or processes associated with tradeoffs between competing demands, such as reproduction and aging, epigenetic processes help researchers go beyond 'black box' explanations (Flatt & Heyland, 2011). Furthermore, hypothesis-generating approaches have the potential to identify relationships beyond those predicted by conventional theories. For example, while the immunocompetence handicap hypothesis (ICHH) for costs of T in males is a an elegant idea that has gained much attention, it has not been well supported empirically and may be over simplistic for human males. Epigenome-wide associations may provide new ways of operationalizing tradeoffs within the immune system, or between other untheorized functions. Candidate genes or pathways uncovered in life history research can then be tested experimentally using pharmacological or genetic engineering using in vitro cell culture or in model organisms.

Broader questions in life history theory

One of the overarching goals of this dissertation has been to develop a framework for studying the proximate mechanisms underlying tradeoffs between reproduction and healthy aging. A central assumption behind this work, laid out in the beginning, is that tradeoffs arise due to conflicts in energy allocation between competing physiological demands favoring either reproduction or maintenance (Kirkwood, 1977; S. C. Stearns, 1989). While intuitively appealing and theoretically plausible, the evidence for such a

direct role of ‘energy’ in somatic maintenance and aging has been surprisingly weak or inconsistent. Energy is not a limiting factor in many contemporary human populations in which costs of reproduction in the form of all-cause mortality and disease prevalence have been observed (e.g. Grundy, 2009; Lv et al., 2015; Y. Zeng et al., 2016). Even in low resource settings with high infectious disease burdens, women appear to recoup the energetic costs of reproduction over time (M. Gurven et al., 2016). Our findings agree with this, as our measure of resource availability did not moderate the effect of gravidity on cellular aging (Chapter 3; Ryan et al., 2018). Similarly, very few of the differentially methylated sites we uncovered during pregnancy were directly tied to processes involved in cellular respiration or catabolism, as might be expected if the dominant source of reproductive costs in women is purely energetic.

While it is important to remain cautious when inferring what may be tissue specific changes from DNAm in blood, there are other reasons to be wary of allusions to energetic trade-offs as a satisfactory mechanistic explanation of the primary driver of CoR. One example of this can be found in research on caloric restriction (CR). CR is the most reliable means of reducing disease and extending lifespan, in some cases by as much as 50% (Heilbronn & Ravussin, 2003; Most, Tosti, Redman, & Fontana, 2017). However, the effect of CR on health and lifespan—in a direction opposite to that of reproduction—seems to contradict the disposable soma theory and challenges the idea that energy availability is central to the aging process. How can energy set the pace of biological aging if reproduction and caloric restriction have such different effects on health and lifespan? One possibility is that CR slows aging by drawing more energy

away from reproductive function and allocating it to bodily maintenance. However, while severe CR of 40% or more does suppress fertility, moderate CR ~20-30% prolongs both longevity and reproductive lifespan (Garcia et al., 2019; Moatt, Nakagawa, Lagisz, & Walling, 2016) which runs counter to the hypothesis that reproduction comes at the expense of maintenance when energy is limited.

Similar questions arise through the links between exercise, energy expenditure, and longevity. Physical activity typically increases resting metabolic rate (J. R. Speakman & Selman, 2003), which comprises the largest contribution to daily energy expenditure (Manini, 2010). Instead of reducing fertility, health, or lifespan, however, exercise improves health and reduces mortality risk (Reimers, Knapp, & Reimers, 2012), contrary to a model of life history tradeoffs based solely on energy availability and consumption. Furthermore, researchers have observed a negative relationship between resting metabolic rate and lifespan in mice, which appears to be confounded by body fat mass – a measure of energy stores (Duarte & Speakman, 2014). These findings are consistent with both ‘rate of living’ and ‘free-radical’ theories of aging (Duarte & Speakman, 2014), but do not fit comfortably within life history tradeoffs whereby energy itself is the limiting resource needed for somatic maintenance, repair, and longevity.

How do we rectify what appear to be contradictions between a fundamental concept in life history theory – tradeoffs between reproduction and aging – and empirical data from other sources? Given well-documented evidence for such tradeoffs both across and within species, it would be premature to discard these ideas entirely. Williams highlighted that genes that benefit fitness do not necessarily improve health or prolong

lifespan, noting that they could have pleiotropic effects on other functions that affect longevity. And Kirkwood moved us beyond genetically hard-wired tradeoffs between early fecundity and late life survival towards a more ecologically oriented framework that allows for individual life history strategies to play out. Both appear to be key to understanding the link between reproduction and aging. However, life history research may need to push beyond the black box that still forms the basis for discussions about tradeoffs in life history traits. More nuanced theories that move beyond 'energy' – and start to operationalize the functional processes that underlie both reproduction and aging using concrete mechanistic insights – may be needed. Epigenetic processes could help us move towards this goal.

Part of my long-term research plans will involve the use of mice or other non-human organisms to test our findings from human populations. One of these projects was recently selected for review for the Banting Postdoctoral Fellowship as part of the Canadian Institutes for Health Research (CIHR). If successful, this project will be carried out in conjunction with Dr. Meaghan Jones at the University of Manitoba and involves an experimental approach to studying the tradeoff between reproduction and aging in mice. Specifically, I will examine the paradoxical role of energy in aging and lifespan by pitting the disposable soma theory against caloric restriction – the only reliable means of prolonging lifespan in numerous species. I aim to address several key questions that are difficult to study in humans: How can energy set the pace of biological aging if reproduction and caloric restriction have such different effects on health and lifespan? Does the energy needed for reproduction come from organs like the heart or the

kidneys, causing these tissues to age faster? Or do mothers with many offspring reduce how much energy they allocate to individual young, handing down an ‘energetic debt’ to the next generation? The goal is to understand how reproduction and caloric restriction – distinct processes that draw energy away from bodily maintenance – impact aging across tissues and generations. Addressing these questions will tell us more about the role of energy in the aging process, which can be used to understand and promote healthy aging across the life course.

If energy itself is not the currency of life history tradeoffs, what is? Perhaps functional constraints – conceived as narrowly as molecular processes and as broadly as physiological set-points – are key. At the molecular scale, proteins, RNAs, and transcriptional machinery do not operate in an infinite functional space, but can alter, silence, or interfere with functional signals from the others. This can create regulatory constraints through which tradeoffs between hormone production, growth, proteostasis, or genomic stability must operate. For example, heat-shock protein-90 is a chaperone vital for signal transduction and protein folding and stability (Erlejman, Lagadari, Toneatto, Piwien-Pilipuk, & Galigniana, 2014). However, it is also required in epigenetic silencing transposable elements through its role in the PIWI-piRNA loading pathway. Hsp90 expression appears to be constrained by the cytotoxic effects of Hsp90 accumulation (Casanueva, Burga, & Lehner, 2012; Feder & Hofmann, 1999; Krebs & Loeschcke, 1994), resulting in a functional tradeoff between cellular buffering and maintaining genomic and epigenomic stability (Ryan et al., 2016, 2018). Other complex molecular networks needed for cell signaling, metabolism, housekeeping, as well as

tissue-specific functions could create functional constraints that are largely decoupled from energetic measures.

If organs do not share identical endocrinological, immunological, or metabolic optima, functional constraints could also arise between tissues. Early in life, optima for tissues not involved in reproduction may be favored over reproductive tissues, but this could reverse at sexual maturity. Alternatively, the physiological setpoints needed for development and maintenance of reproductive tissues during pregnancy and breastfeeding may pull other non-reproductive tissues outside of their ideal operating range. The poorer efficiency and additional 'friction' caused by non-reproductive organs operating outside their optima could lead to the accumulation of waste or toxic by-products such as reactive oxygen species in these tissues. For example, the greatly expanded blood volume during pregnancy (up to 100%) may be especially taxing to both the cardiovascular and renal system. Findings like this could help to explain the heterogeneous effects of parity on women's health, with diseases for somatic organs, such as the heart, kidneys, and bones often increasing with parity, while cancers of the ovaries, endometrium, and breast tend to decrease. Such tradeoffs *between tissues* may lead to 'aging mosaicism' between organs, a testable hypothesis using pan-tissue biomarkers of epigenetic age (Horvath, 2013). For these and other reasons, it is also important to be cautious when interpreting the significance of DNAm differences in one tissue (i.e. blood) meant to capture biological functions in another (i.e. brain). While concordance between tissues does exist for some CpG sites, this is often not the case (Edgar et al., 2017).

Tradeoffs – energetic or functional – are usually thought of as an individual, intra-generational phenomenon. There is no reason, however, that tradeoffs cannot occur inter-generationally, such as between a mother and her young. Intragenerational costs of reproduction can arise because parents and offspring differ genetically (Trivers, 1972), and would be selected for when a mother stands to benefit from hedging her bets between current reproductive investment and her residual reproductive value (Williams, 1966b). A mother who is energetically constrained may still opt to reproduce while conserving her own maintenance effort by passing the energetic debt onto her young. In theory, this could curtail offspring developmental potential, reducing total somatic capacity and eventually accelerating biological aging. Evidence from non-human animals suggests that maternal age and caloric restriction can both have negative impacts on offspring cognition, behavior (Sampino et al., 2017), or lifespan (M. J. Bock, Jarvis, Corey, Stone, & Gribble, 2019). In humans, advanced parental age is associated with improved offspring mortality outcomes (Carslake, Tynelius, van den Berg, & Davey Smith, 2019). However, this appears to be because social factors outweigh any developmental or physiological disadvantages, since older parents are more likely to be healthier and wealthier than their younger counterparts (Carslake et al., 2019). These findings highlight the importance of social status and support – which feed into education, diet, exercise, and stress, and are poorly described by energy expenditure – when studying costs of reproduction in either mothers or their offspring.

The social dimensions of tradeoffs between reproduction and somatic maintenance may play out in other ways. For example, breastfeeding is often the most energetically costly

component of reproduction in women, but is a social as well as a physiological process. The timing, duration, and intensity of breastfeeding will influence energetic demands upon the mother (Hinde & Milligan, 2011), yet are shaped by women's socioeconomic status, social support system, and opportunities to breastfeed. Furthermore, the energetic and functional role of breastmilk changes through time, meaning that from both energetic and functional perspectives breastfeeding is not a uniform process that can be easily categorized. This heterogeneity can be seen in the relatively small and loosely tied networks that we observed for breastfeeding compared to pregnancy (Chapter 4). The measures of breastfeeding in the CLHNS are relatively crude and were not included due to the rapid increase in parameters needed to model them. Incorporating factors such as infant age, feeding frequency, and supplementary feeding with the full cohort sample will help us to refine our understanding about how and where the maternal methylome responds to lactation in women, and what this might tell us about women's long term health and aging. Similar opportunities exist to refine our understanding of costs of reproduction may come from considering the importance of stage of pregnancy, which may be operationalized by trimester or weeks since conception.

Better measures of social context will also be necessary to fully make use of our data on the costs of reproduction in men. Partnership and fatherhood, for example, are likely to be important in male health and aging, as evidenced by epidemiological studies showing higher mortality among single and childless men. Unfortunately, our preliminary sample only consisted of 11 men who were married, and 9 men with children, limiting

our ability to study costs of reproduction in men within the context of their social roles as fathers and husbands. The larger sample sizes we have planned will help us to gain better resolution on the importance of men's sociobiological roles in their epigenetic aging and health. Samples from a second time point will also give us a window in men's epigenome later in life. This is likely to be important for both physiological and social reasons. Men continue to develop and build muscle into their late 20's, and any costs of parenting on men's health may become more apparent as children age over time. Whether or not men show similar effects of gravidity or parity as their partners is an interesting question that could also help to rule out some of the social factors that might confound our findings.

Conclusion

Nearly a decade and a half ago, Morange stated that "biological research is at a crossroads between reductionism and holism" (Morange, 2006), which is a tension that biological anthropology and human biology have grappled with for many decades. Epigenetic processes promise to help bridge the gap between these two worlds. They provide us with new conceptual tools for thinking about biological processes and change, and new methods for studying them. By their very nature, epigenetic processes make us think interdisciplinarily, which is one of the strengths of biological anthropology. While ideas about plasticity and sensitivity to the environment come with their own theoretical and ethical dilemmas, the biological anthropologists and human biologists of the 21st century are in a powerful position to make important headway in longstanding questions about tradeoffs in health and evolution.

References

Adair, L. S., Popkin, B. M., Akin, J. S., Guilkey, D. K., Gultiano, S., Borja, J., … Hindin, M. J. (2011). Cohort Profile: The Cebu Longitudinal Health and Nutrition Survey. *International Journal of Epidemiology*, *40*(3), 619–625. https://doi.org/10.1093/ije/dyq085

Adam, E. K., Klimes-Dougan, B., & Gunnar, M. R. (2007). Emotional and social regulation of the adrenocortical response to stress. In D. Coch, G. Dawson, & K. W. Fischer (Eds.), *Human behavior and the developing brain: Atypical development*. New York, NY: Guilford Press.

Adkins-Regan, E. (2005). *Hormones and animal social behavior*. Princeton, NJ: Princeton University Press.

Admyre, C., Johansson, S. M., Qazi, K. R., Filén, J.-J., Lahesmaa, R., Norman, M., … Gabrielsson, S. (2007). Exosomes with Immune Modulatory Features Are Present in Human Breast Milk. *The Journal of Immunology*, *179*(3), 1969–1978. https://doi.org/10.4049/jimmunol.179.3.1969

Affolter, M., Grass, L., Vanrobaeys, F., Casado, B., & Kussmann, M. (2010). Qualitative and quantitative profiling of the bovine milk fat globule membrane proteome. *Journal of Proteomics*, *73*(6), 1079–1088. https://doi.org/10.1016/j.jprot.2009.11.008

Aghaeepour, N., Ganio, E. A., Mcilwain, D., Tsai, A. S., Tingle, M., Gassen, S. V., … Gaudilliere, B. (2017). An immune clock of human pregnancy. *Science Immunology*, *2*(15), eaan2946. https://doi.org/10.1126/sciimmunol.aan2946

Albrecht, E. D., & Pepe, G. J. (2015). Chapter 40—Placental Endocrine Function and Hormone Action. In T. M. Plant & A. J. Zeleznik (Eds.), *Knobil and Neill's Physiology of Reproduction (Fourth Edition)* (pp. 1783–1834). San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-397175-3.00040-5

Allis, C. D., Caparros, M.-L., Jenuwein, T., & Reinberg, D. (Eds.). (2015). *Epigenetics* (Second edition). Cold Spring Harbor, New York: CSH Press, Cold Spring Harbor Laboratory Press.

Alvarado, L. C., Muller, M. N., Emery Thompson, M., Klimek, M., Nenko, I., & Jasienska, G. (2015). The Paternal Provisioning Hypothesis: Effects of workload and testosterone production on men's musculature. *American Journal of Physical Anthropology*, *158*(1), 19–35. https://doi.org/10.1002/ajpa.22771

Amster, G., Murphy, D. A., Milligan, W. R., & Sella, G. (2020). Changes in life history and population size can explain the relative neutral diversity levels on X and autosomes in extant human populations. *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.1915664117

Anderson, S. M., MacLean, P. S., McManaman, J. L., & Neville, M. C. (2015). Chapter 46—Lactation and its Hormonal Control. In T. M. Plant & A. J. Zeleznik (Eds.), *Knobil and Neill's Physiology of Reproduction (Fourth Edition)* (pp. 2055–2105). San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-397175-3.00046-6

Anton, A. L. (2016). Aging in Classical Philosophy. In *The Palgrave Handbook of the Philosophy of Aging* (pp. 115–134). Springer.

Arathimos, R., Sharp, G. C., Granell, R., Tilling, K., & Relton, C. L. (2018). Associations of sex hormone-binding globulin and testosterone with genome-wide DNA methylation. *BMC Genetics*, *19*. https://doi.org/10.1186/s12863-018-0703-y

Ardia, D. R., Schat, K. A., & Winkler, D. W. (2003). Reproductive effort reduces long-term immune function in breeding tree swallows (Tachycineta bicolor). *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1525), 1679–1683. https://doi.org/10.1098/rspb.2003.2424

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., … Sherlock, G. (2000, May 1). Gene Ontology: Tool for the unification of biology [Comments and Opinion]. https://doi.org/10.1038/75556

Astle, W. J., Elding, H., Jiang, T., Allen, D., Ruklisa, D., Mann, A. L., … Soranzo, N. (2016). The Allelic Landscape of Human Blood Cell Trait Variation and Links to Common Complex Disease. *Cell*, *167*(5), 1415-1429.e19. https://doi.org/10.1016/j.cell.2016.10.042

Auclair, G., & Weber, M. (2012). Mechanisms of DNA methylation and demethylation in mammals. *Biochimie*, *94*(11), 2202–2211. https://doi.org/10.1016/j.biochi.2012.05.016

Aviv, A., Valdes, A., Gardner, J. P., Swaminathan, R., Kimura, M., & Spector, T. D. (2006). Menopause Modifies the Association of Leukocyte Telomere Length with Insulin Resistance and Inflammation. *J Clin Endocrinol Metab*, *91*(2), 635–640.

Aydın, M. Ş., Yiğit, E. N., Vatandaşlar, E., Erdoğan, E., & Öztürk, G. (2018). Transfer and Integration of Breast Milk Stem Cells to the Brain of Suckling Pups. *Scientific Reports*, *8*(1), 1–9. https://doi.org/10.1038/s41598-018-32715-5

Bakaysa, S. L., Mucci, L. A., Slagboom, P. E., Boomsma, D. I., McClearn, G. E., Johansson, B., & Pedersen, N. L. (2007). Telomere length predicts survival independent of genetic influences. *Aging Cell*, *6*(6), 769–774.

Bancroft, J. (2012). The behavioral correlates of testosterone. In Eberhard Nieschlag, H. M. Behre, & S. Nieschlag (Eds.), *Testosterone: Action, deficiency, substitution* (4ed., pp. 87–122). Cambridge, UK: Cambridge University Press.

Baranzini, S. E., Srinivasan, R., Khankhanian, P., Okuda, D. T., Nelson, S. J., Matthews, P. M., … Pelletier, D. (2010). Genetic variation influences glutamate concentrations in brains of patients with multiple sclerosis. *Brain*, *133*(9), 2603–2611. https://doi.org/10.1093/brain/awq192

Bardanzellu, F., Fanos, V., & Reali, A. (2017). “Omics” in Human Colostrum and Mature Milk: Looking to Old Data with New Eyes. *Nutrients*, *9*(8), 843. https://doi.org/10.3390/nu9080843

Barha, C. K., Hanna, C. W., Salvante, K. G., Wilson, S. L., Robinson, W. P., Altman, R. M., & Nepomnaschy, P. A. (2016). Number of Children and Telomere Length in Women: A Prospective, Longitudinal Evaluation. *PLOS ONE*, *11*(1), e0146424. https://doi.org/10.1371/journal.pone.0146424

Bar-Sadeh, B., Rudnizky, S., Pnueli, L., Bentley, G. R., Stöger, R., Kaplan, A., & Melamed, P. (2020). Unravelling the role of epigenetics in reproductive

adaptations to early-life environment. *Nature Reviews Endocrinology*, 1–15. https://doi.org/10.1038/s41574-020-0370-8

Bateson, W. (1913). *Mendel's principles of heredity*. At the University Press.

Bauer, K. A. (2014). Hematologic changes in pregnancy. *UpToDate*.

Beach, S. R., Dogan, M. V., Lei, M., Cutrona, C. E., Gerrard, M., Gibbons, F. X., … Philibert, R. A. (2015). Methylomic aging as a window onto the influence of lifestyle: Tobacco and alcohol use alter the rate of biological aging. *Journal of the American Geriatrics Society*, *63*(12), 2519–2525.

Beeri, M. S., Rapp, M., Schmeidler, J., Reichenberg, A., Purohit, D. P., Perl, D. P., … Silverman, J. M. (2009). Number of children is associated with neuropathology of Alzheimer's disease in women. *Neurobiology of Aging*, *30*(8), 1184–1191. https://doi.org/10.1016/j.neurobiolaging.2007.11.011

Behl, C., Skutella, T., Lezoualc'H, F., Post, A., Widmann, M., Newton, C. J., & Holsboer, F. (1997). Neuroprotection against Oxidative Stress by Estrogens: Structure-Activity Relationship. *Molecular Pharmacology*, *51*(4), 535–541. https://doi.org/10.1124/mol.51.4.535

Belsky, D. W., Caspi, A., Arseneault, L., Baccarelli, A., Corcoran, D. L., Gao, X., … Moffitt, T. E. (2020). Quantification of the pace of biological aging in humans through a blood test, the DunedinPoAm DNA methylation algorithm. *ELife*, *9*, e54870. https://doi.org/10.7554/eLife.54870

Belsky, D. W., Moffitt, T. E., Cohen, A. A., Corcoran, D. L., Levine, M. E., Prinz, J. A., … Caspi, A. (2018). Eleven Telomere, Epigenetic Clock, and Biomarker-Composite

Quantifications of Biological Aging: Do They Measure the Same Thing? *American Journal of Epidemiology*, *187*(6), 1220–1230. https://doi.org/10.1093/aje/kwx346

Benayoun, B. A., Pollina, E. A., & Brunet, A. (2015). Epigenetic regulation of ageing: Linking environmental inputs to genomic stability. *Nature Reviews Molecular Cell Biology*, *16*(10), 593–610. https://doi.org/10.1038/nrm4048

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, 289–300.

Bennett, N. C., Gardiner, R. A., Hooper, J. D., Johnson, D. W., & Gobe, G. C. (2010). Molecular cell biology of androgen receptor signalling. *The International Journal of Biochemistry & Cell Biology*, *42*(6), 813–827. https://doi.org/10.1016/j.biocel.2009.11.013

Beral, V. (1985). Long term effects of childbearing on health. *Journal of Epidemiology & Community Health*, *39*(4), 343–346. https://doi.org/10.1136/jech.39.4.343

Berg, J. S., Evans, J. P., Leigh, M. W., Omran, H., Bizon, C., Mane, K., … Zariwala, M. A. (2011). Next generation massively parallel sequencing of targeted exomes to identify genetic mutations in primary ciliary dyskinesia: Implications for application to clinical testing. *Genetics in Medicine : Official Journal of the American College of Medical Genetics*, *13*(3), 218–229. https://doi.org/10.1097/GIM.0b013e318203cff2

Bethancourt, H. J., Kratz, M., Hayes, M. G., Kuzawa, C. W., Borja, J. B., Duazo, P. L., … Eisenberg, D. T. A. (2015). No Association between Blood Telomere Length and Longitudinally-Assessed Diet or Adiposity or Diet in a Young Adult Filipino Population. *European Journal of Nutrition*, 1–14.

Bhasin, S., Jasuja, R., Serra, C., Singh, R., Storer, T. W., Guo, W., … Basaria, S. (2012). Androgen effects on the skeletal muscle. In Eberhard Nieschlag, H. M. Behre, & S. Nieschlag (Eds.), *Testosterone: Action, deficiency, substitution* (4ed., pp. 191–206). Cambridge, UK: Cambridge University Press.

Bibikova, M., Barnes, B., Tsan, C., Ho, V., Klotzle, B., Le, J. M., … Shen, R. (2011). High density DNA methylation array with single CpG site resolution. *Genomics*, *98*(4), 288–295. https://doi.org/10.1016/j.ygeno.2011.07.007

Biesecker, L. G. (2013). Hypothesis-generating research and predictive medicine. *Genome Research*, *23*(7), 1051–1053. https://doi.org/10.1101/gr.157826.113

Bigiu, N., & Pandi, B. (2015). Maternal depletion syndrome. *Gineco.Eu*, *11*(40), 6.

Bilder, G. E. (2016). *Human biological aging: From macromolecules to organ systems* (1st edition). Hoboken, New Jersey: Wiley-Blackwell.

Binder, A. M., Corvalan, C., Mericq, V., Pereira, A., Santos, J. L., Horvath, S., … Michels, K. B. (2018). Faster ticking rate of the epigenetic clock is associated with faster pubertal development in girls. *Epigenetics*, *13*(1), 85–94. https://doi.org/10.1080/15592294.2017.1414127

Blackburn, E. H., & Gall, J. G. (1978). A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. *Journal of Molecular Biology*, *120*(1), 33–53.

Blouin, K., Boivin, A., & Tchernof, A. (2008). Androgens and body fat distribution. *The Journal of Steroid Biochemistry and Molecular Biology*, *108*(3), 272–280. https://doi.org/10.1016/j.jsbmb.2007.09.001

Boas, F. (1912). Changes in the Bodily Form of Descendants of Immigrants. *American Anthropologist*, *14*(3), 530–562. JSTOR. Retrieved from JSTOR.

Boas, F., & Boas, H. M. (1913). The head-forms of the Italians as influenced by heredity and environment. *American Anthropologist*, *15*(2), 163–188.

Bobrow, K. L., Quigley, M. A., Green, J., Reeves, G. K., & Beral, V. (2013). Persistent effects of women's parity and breastfeeding patterns on their body mass index: Results from the Million Women Study. *International Journal of Obesity*, *37*(5), 712–717. https://doi.org/10.1038/ijo.2012.76

Bock, C. (2012). Analysing and interpreting DNA methylation data. *Nature Reviews Genetics*, *13*(10), 705–719. https://doi.org/10.1038/nrg3273

Bock, M. J., Jarvis, G. C., Corey, E. L., Stone, E. E., & Gribble, K. E. (2019). Maternal age alters offspring lifespan, fitness, and lifespan extension under caloric restriction. *Scientific Reports*, *9*(1), 1–16. https://doi.org/10.1038/s41598-019-40011-z

Bocklandt, S., Lin, W., Sehl, M. E., Sánchez, F. J., Sinsheimer, J. S., Horvath, S., & Vilain, E. (2011). Epigenetic Predictor of Age. *PLoS ONE*, *6*(6). https://doi.org/10.1371/journal.pone.0014821

Boddy, A. M., Fortunato, A., Sayres, M. W., & Aktipis, A. (2015). Fetal microchimerism and maternal health: A review and evolutionary analysis of cooperation and conflict beyond the womb. *BioEssays*, *37*(10), 1106–1118. https://doi.org/10.1002/bies.201500059

Bohannon, R. W. (2008). Hand-grip dynamometry predicts future outcomes in aging adults. *Journal of Geriatric Physical Therapy*, *31*(1), 3–10.

Boks, M. P., Mierlo, H. C. van, Rutten, B. P. F., Radstake, T. R. D. J., De Witte, L., Geuze, E., … Vermetten, E. (2015). Longitudinal changes of telomere length and epigenetic age related to traumatic stress and post-traumatic stress disorder. *Psychoneuroendocrinology*, *51*, 506–512. https://doi.org/10.1016/j.psyneuen.2014.07.011

Bollati, V., Schwartz, J., Wright, R., Litonjua, A., Tarantini, L., Suh, H., … Baccarelli, A. (2009). Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mechanisms of Ageing and Development*, *130*(4), 234–239. https://doi.org/10.1016/j.mad.2008.12.003

Bolund, E. (2020). The challenge of measuring trade-offs in human life history research. *Evolution and Human Behavior*, S1090513820301185. https://doi.org/10.1016/j.evolhumbehav.2020.09.003

Bolund, E., Lummaa, V., Smith, K. R., Hanson, H. A., & Maklakov, A. A. (2016). Reduced costs of reproduction in females mediate a shift from a male-biased to a female-biased lifespan in humans. *Scientific Reports*, *6*, 24672. https://doi.org/10.1038/srep24672

Bourgon, R., Gentleman, R., & Huber, W. (2010). Independent filtering increases detection power for high-throughput experiments. *Proceedings of the National Academy of Sciences*, *107*(21), 9546–9551. https://doi.org/10.1073/pnas.0914005107

Boveri, T. (1902). Uber mehrpolige Mitosen als Mittle zur Analyse des Zellkerns. *Verhandl Phys-Med Ges (Wulzburg) NF*, *35*, 67–90.

Bowler, P. J. (2003). *Evolution: The History of an Idea*. Berkeley, CA: University of California Press.

Boyce, W. T., & Kobor, M. S. (2015). Development and the epigenome: The 'synapse' of gene-environment interplay. *Developmental Science*, *18*(1), 1–23. https://doi.org/10.1111/desc.12282

Breitling, L. P., Saum, K.-U., Perna, L., Schöttker, B., Holleczek, B., & Brenner, H. (2016). Frailty is associated with the epigenetic clock but not with telomere length in a German cohort. *Clinical Epigenetics*, *8*(1), 21. https://doi.org/10.1186/s13148-016-0186-5

Bribiescas, R. G. (2001). Reproductive ecology and life history of the human male. *American Journal of Physical Anthropology*, *116*(S33), 148–176. https://doi.org/10.1002/ajpa.10025

Bribiescas, R. G. (2016). *How Men Age: What Evolution Reveals about Male Health and Mortality*. Princeton, New Jersey: Princeton University Press.

Bult, C. J., Blake, J. A., Smith, C. L., Kadin, J. A., Richardson, J. E., Group, the M. G. D., … Zhu, Y. (2019). Mouse Genome Database (MGD) 2019. *Nucleic Acids Research*, *47*(D1), D801–D806. https://doi.org/10.1093/nar/gky1056

Bulterijs, S., Hull, R. S., Björk, V. C. E., & Roy, A. G. (2015). It is time to classify biological aging as a disease. *Frontiers in Genetics*, *6*. https://doi.org/10.3389/fgene.2015.00205

Busato, F., Dejeux, E., Gut, I. G., & Tost, J. (2018). Quantitative DNA methylation analysis at single-nucleotide resolution by pyrosequencing®. In J. Tost (Ed.), *DNA Methylation Protocols* (pp. 427–445). New York, NY: Springer.

Butte, N. F., & King, J. C. (2005). Energy requirements during pregnancy and lactation. *Public Health Nutrition*, *8*(7a). https://doi.org/10.1079/PHN2005793

Campbell, K. H. S., McWhir, J., Ritchie, W. A., & Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature*, *380*(6569), 64. https://doi.org/10.1038/380064a0

Carja, O., MacIsaac, J. L., Mah, S. M., Henn, B. M., Kobor, M. S., Feldman, M. W., & Fraser, H. B. (2017). Worldwide patterns of human epigenetic variation. *Nature Ecology & Evolution*, *1*(10), 1577–1583. https://doi.org/10.1038/s41559-017-0299-z

Carrero, J. J., Stenvinkel, P., Fellstrom, B., Qureshi, A. R., Lamb, K., Heimburger, O., … Shiels, P. G. (2008). Telomere attrition is associated with inflammation, low

fetuin-A levels and high mortality in prevalent haemodialysis patients. *Journal of Internal Medicine*, *263*(3), 302–312.

Carslake, D., Tynelius, P., van den Berg, G. J., & Davey Smith, G. (2019). Associations of parental age with offspring all-cause and cause-specific adult mortality. *Scientific Reports*, *9*(1), 17097. https://doi.org/10.1038/s41598-019-52853-8

Casanueva, M. O., Burga, A., & Lehner, B. (2012). Fitness Trade-Offs and Environmentally Induced Mutation Buffering in Isogenic C. elegans. *Science*, *335*(6064), 82–85. https://doi.org/10.1126/science.1213491

Cawthon, R. M., Smith, K. R., O'Brien, E., Sivatchenko, A., & Kerber, R. A. (2003). Association between telomere length in blood and mortality in people aged 60 years or older. *The Lancet*, *361*(9355), 393–395.

Chen, B. H., Marioni, R. E., Colicino, E., Peters, M. J., Ward-Caviness, C. K., Tsai, P.-C., … Guan, W. (2016). DNA methylation-based measures of biological age: Meta-analysis predicting time to death. *Aging (Albany NY)*, *8*(9), 1844.

Childebayeva, A., Harman, T., Weinstein, J., Goodrich, J. M., Dolinoy, D. C., Day, T. A., … Brutsaert, T. D. (2019). DNA Methylation Changes Are Associated With an Incremental Ascent to High Altitude. *Frontiers in Genetics*, *10*, 1062. https://doi.org/10.3389/fgene.2019.01062

Chiou, K. L., Montague, M. J., Goldman, E. A., Watowich, M. M., Sams, S. N., Song, J., … Snyder-Mackler, N. (2020). Rhesus macaques as a tractable physiological model of human ageing. *Philosophical Transactions of the Royal Society B:*

Biological Sciences, *375*(1811), 20190612. https://doi.org/10.1098/rstb.2019.0612

Cho, J. I., Basnyat, B., Jeong, C., Di Rienzo, A., Childs, G., Craig, S. R., … Beall, C. M. (2017). Ethnically Tibetan women in Nepal with low hemoglobin concentration have better reproductive outcomes. *Evolution, Medicine, and Public Health*, *2017*(1), 82–96. https://doi.org/10.1093/emph/eox008

Christiansen, L., Lenart, A., Tan, Q., Vaupel, J. W., Aviv, A., McGue, M., & Christensen, K. (2016). DNA methylation age is associated with mortality in a longitudinal Danish twin study. *Aging Cell*, *15*(1), 149–154. https://doi.org/10.1111/acel.12421

Clark, W. R. (2002). *A means to an end: The biological basis of aging and death*. New York, NY: Oxford University Press.

Clutton-Brock, T. H. (1989). Mammalian mating systems. *Proceedings of the Royal Society of London. B. Biological Sciences*, *236*(1285), 339–372. https://doi.org/10.1098/rspb.1989.0027

Coitinho, D. C., Sichieri, R., & Benício, M. H. D. (2001). Obesity and weight change related to parity and breast-feeding among parous women in Brazil. *Public Health Nutrition*, *4*(4), 865–870. https://doi.org/10.1079/PHN2001125

Corbett, S., Courtiol, A., Lummaa, V., Moorad, J., & Stearns, S. (2018). The transition to modernity and chronic disease: Mismatch and natural selection. *Nature Reviews Genetics*, 1. https://doi.org/10.1038/s41576-018-0012-3

Crews, D. E. (2003). *Human senescence: Evolutionary and biocultural perspectives* (Vol. 36). Cambridge, UK: Cambridge University Press.

Crick, F. H., Barnett, L., Brenner, S., & Watts-Tobin, R. J. (1961). General nature of the genetic code for proteins. *Nature*, *192*(4809), 1227–1232.

Crimmins, E. M., Shim, H., Zhang, Y. S., & Kim, J. K. (2019). Differences between Men and Women in Mortality and the Health Dimensions of the Morbidity Process. *Clinical Chemistry*, *65*(1), 135–145. https://doi.org/10.1373/clinchem.2018.288332

Croteau-Chonka, D. C., Marvelle, A. F., Lange, E. M., Lee, N. R., Adair, L. S., Lange, L. A., & Mohlke, K. L. (2011). Genome-wide association study of anthropometric traits and evidence of interactions with age and study year in Filipino women. *Obesity*, *19*(5), 1019–1027.

Croteau-Chonka, D. C., Wu, Y., Li, Y., Fogarty, M. P., Lange, L. A., Kuzawa, C. W., … Mohlke, K. L. (2012). Population-specific coding variant underlies genome-wide association with adiponectin level. *Hum Mol Genet*, *21*(2), 463–471.

Curtsinger, J. W., Fukui, H. H., Khazaeli, A. A., Kirscher, A., Pletcher, S. D., Promislow, D. E., & Tatar, M. (1995). Genetic variation and aging. *Annu Rev Genet*, *29*(1), 553–575.

Dabbs, J. M., & Morris, R. (1990). Testosterone, Social Class, and Antisocial Behavior in a Sample of 4,462 Men. *Psychological Science*, *1*(3), 209–211. https://doi.org/10.1111/j.1467-9280.1990.tb00200.x

Dahly, D. L., & Adair, L. S. (2007). Quantifying the urban environment: A scale measure of urbanicity outperforms the urban–rural dichotomy. *Social Science & Medicine*, *64*(7), 1407–1419.

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D., & Milzani, A. (2006). Biomarkers of Oxidative Damage in Human Disease. *Clinical Chemistry*, *52*(4), 601–623. https://doi.org/10.1373/clinchem.2005.061408

Darwin, C. (1859). *The Origin Of Species* (150th Anniversary Edition). New York, NY: Signet Classic.

Darwin, C., & Wallace, A. (1858). On the tendency of species to form varieties; and on the perpetuation of varieties and species by natural means of selection. *Journal of the Proceedings of the Linnean Society of London. Zoology*, *3*(9), 45–62.

Davis, E. G., Humphreys, K. L., McEwen, L. M., Sacchet, M. D., Camacho, M. C., MacIsaac, J. L., … Gotlib, I. H. (2017). Accelerated DNA methylation age in adolescent girls: Associations with elevated diurnal cortisol and reduced hippocampal volume. *Translational Psychiatry*, *7*(8), e1223–e1223. https://doi.org/10.1038/tp.2017.188

Dawkins, R. (2016). *The selfish gene*. Oxford university press.

de Goede, O. M., Lavoie, P. M., & Robinson, W. P. (2016). Characterizing the hypomethylated DNA methylation profile of nucleated red blood cells from cord blood. *Epigenomics*, *8*(11), 1481–1494. https://doi.org/10.2217/epi-2016-0069

de Lange, A.-M. G., Kaufmann, T., van der Meer, D., Maglanoc, L. A., Alnæs, D., Moberget, T., … Westlye, L. T. (2019). Population-based neuroimaging reveals traces of childbirth in the maternal brain. *Proceedings of the National Academy of Sciences*, *116*(44), 22341–22346. https://doi.org/10.1073/pnas.1910666116

Deans, C., & Maggert, K. A. (2015). What Do You Mean, “Epigenetic”? *Genetics*, *199*(4), 887–896. https://doi.org/10.1534/genetics.114.173492

Demerath, E. W., Guan, W., Grove, M. L., Aslibekyan, S., Mendelson, M., Zhou, Y.-H., … Boerwinkle, E. (2015). Epigenome-wide association study (EWAS) of BMI, BMI change and waist circumference in African American adults identifies multiple replicated loci. *Human Molecular Genetics*, *24*(15), 4464–4479. https://doi.org/10.1093/hmg/ddv161

Doblhammer, G., & Oeppen, J. (2003). Reproduction and longevity among the British peerage: The effect of frailty and health selection. *Proceedings of the Royal Society of London B: Biological Sciences*, *270*(1524), 1541–1547. https://doi.org/10.1098/rspb.2003.2400

Dreher, J.-C., Dunne, S., Pazderska, A., Frodl, T., Nolan, J. J., & O’Doherty, J. P. (2016). Testosterone causes both prosocial and antisocial status-enhancing behaviors in human males. *Proceedings of the National Academy of Sciences*, 201608085. https://doi.org/10.1073/pnas.1608085113

Dribe, M. (2004). Long-term effects of childbearing on mortality: Evidence from pre-industrial Sweden. *Population Studies*, *58*(3), 297–310.

Du, P., Kibbe, W. A., & Lin, S. M. (2008). lumi: A pipeline for processing Illumina microarray. *Bioinformatics*, *24*(13), 1547–1548.

Du, P., Zhang, X., Huang, C.-C., Jafari, N., Kibbe, W. A., Hou, L., & Lin, S. M. (2010). Comparison of Beta-value and M-value methods for quantifying methylation

levels by microarray analysis. *BMC Bioinformatics*, *11*, 587. https://doi.org/10.1186/1471-2105-11-587

Duarte, L. C., & Speakman, J. R. (2014). Low resting metabolic rate is associated with greater lifespan because of a confounding effect of body fatness. *Age (Dordrecht, Netherlands)*, *36*(6), 9731. https://doi.org/10.1007/s11357-014-9731-3

Dugué, P.-A., English, D. R., MacInnis, R. J., Jung, C.-H., Bassett, J. K., FitzGerald, L. M., … Milne, R. L. (2016). Reliability of DNA methylation measures from dried blood spots and mononuclear cells using the HumanMethylation450k BeadArray. *Scientific Reports*, *6*, 30317. https://doi.org/10.1038/srep30317

Duncan, E. J., Gluckman, P. D., & Dearden, P. K. (2014). Epigenetics, plasticity, and evolution: How do we link epigenetic change to phenotype? *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *322*(4), 208–220. https://doi.org/10.1002/jez.b.22571

Edgar, R. D., Jones, M. J., Meaney, M. J., Turecki, G., & Kobor, M. S. (2017). BECon: A tool for interpreting DNA methylation findings from blood in the context of brain. *Translational Psychiatry*, *7*(8), e1187. https://doi.org/10.1038/tp.2017.171

Ehrlich, M. (2002). DNA methylation in cancer: Too much, but also too little. *Oncogene*, *21*(35), 5400–5413. https://doi.org/10.1038/sj.onc.1205651

Eichler, E. E., Flint, J., Gibson, G., Kong, A., Leal, S. M., Moore, J. H., & Nadeau, J. H. (2010). Missing heritability and strategies for finding the underlying causes of

complex disease. *Nature Reviews Genetics*, *11*(6), 446–450. https://doi.org/10.1038/nrg2809

Eisen, J. (2010, August 3). The Tree of Life: Human genome project oversold? sure but lets not undersell basic science. Retrieved September 24, 2020, from The Tree of Life website: https://phylogenomics.blogspot.com/2010/08/human-genome-project-oversold-sure-but.html

Eisenberg, D. T. (2016). Telomere length measurement validity: The coefficient of variation is invalid and cannot be used to compare quantitative polymerase chain reaction and Southern blot telomere length measurement techniques. *Int J Epidemiol*, *45*(4), 1295–1298.

Eisenberg, D. T. A., Borja, J. B., Hayes, M. G., & Kuzawa, C. W. (2017). Early life infection, but not breastfeeding, predicts adult blood telomere lengths in the Philippines. *American Journal of Human Biology*, *29*(4).

Eisenberg, D. T., Kuzawa, C. W., & Hayes, M. G. (2015). Improving qPCR telomere length assays: Controlling for well position effects increases statistical power. *Am J Hum Biol*, *27*(4), 570–575.

Eisenegger, C., Haushofer, J., & Fehr, E. (2011). The role of testosterone in social interaction. *Trends in Cognitive Sciences*, *15*(6), 263–271. https://doi.org/10.1016/j.tics.2011.04.008

Ellis, B. J., & Del Giudice, M. (2019). Developmental Adaptation to Stress: An Evolutionary Perspective. *Annual Review of Psychology*, *70*(1), 111–139. https://doi.org/10.1146/annurev-psych-122216-011732

Ellison, Peter T. (1990). Human Ovarian Function and Reproductive Ecology: New Hypotheses. *American Anthropologist*, *92*(4), 933–952. https://doi.org/10.1525/aa.1990.92.4.02a00050

Ellison, Peter Thorpe. (2009). *On fertile ground: A natural history of human reproduction*. Cambridge, MA: Harvard University Press.

Emery Thompson, M. (2013). Comparative Reproductive Energetics of Human and Nonhuman Primates. *Annual Review of Anthropology*, *42*(1), 287–304. https://doi.org/10.1146/annurev-anthro-092412-155530

Emery Thompson, M., Rosati, A. G., & Snyder-Mackler, N. (2020). Insights from evolutionarily relevant models for human ageing. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1811), 20190605. https://doi.org/10.1098/rstb.2019.0605

Erlejman, A. G., Lagadari, M., Toneatto, J., Piwien-Pilipuk, G., & Galigniana, M. D. (2014). Regulatory role of the 90-kDa-heat-shock protein (Hsp90) and associated factors on gene expression. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, *1839*(2), 71–87. https://doi.org/10.1016/j.bbagrm.2013.12.006

Escasa, M. J., Casey, J. F., & Gray, P. B. (2011). Salivary Testosterone Levels in Men at a U.S. Sex Club. *Archives of Sexual Behavior*, *40*(5), 921–926. https://doi.org/10.1007/s10508-010-9711-3

Fagny, M., Patin, E., MacIsaac, J. L., Rotival, M., Flutre, T., Jones, M. J., … Quintana-Murci, L. (2015). The epigenomic landscape of African rainforest hunter-

gatherers and farmers. *Nature Communications*, *6*, 10047. https://doi.org/10.1038/ncomms10047

Fahy, G. M., Brooke, R. T., Watson, J. P., Good, Z., Vasanawala, S. S., Maecker, H., … Horvath, S. (2019). Reversal of epigenetic aging and immunosenescent trends in humans. *Aging Cell*, *18*(6), e13028. https://doi.org/10.1111/acel.13028

Fan, X., Jin, T., Zhao, S., Liu, C., Han, J., Jiang, X., & Jiang, Y. (2015). Circulating CCR7+ICOS+ Memory T Follicular Helper Cells in Patients with Multiple Sclerosis. *PLOS ONE*, *10*(7), e0134523. https://doi.org/10.1371/journal.pone.0134523

Farzaneh-Far, R., Lin, J., Epel, E., Lapham, K., Blackburn, E., & Whooley, M. A. (2010). Telomere length trajectory and its determinants in persons with coronary artery disease: Longitudinal findings from the heart and soul study. *PLoS One*, *5*(1), e8612.

Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology*, *61*(1), 243–282. https://doi.org/10.1146/annurev.physiol.61.1.243

Field, A. E., Robertson, N. A., Wang, T., Havas, A., Ideker, T., & Adams, P. D. (2018). DNA Methylation Clocks in Aging: Categories, Causes, and Consequences. *Molecular Cell*, *71*(6), 882–895. https://doi.org/10.1016/j.molcel.2018.08.008

Finch, C. E. (2009). Update on Slow Aging and Negligible Senescence – A Mini-Review. *Gerontology*, *55*(3), 307–313. https://doi.org/10.1159/000215589

Finch, C. E. (2010). Evolution of the human lifespan and diseases of aging: Roles of infection, inflammation, and nutrition. *Proceedings of the National Academy of Sciences*, *107*(suppl 1), 1718–1724. https://doi.org/10.1073/pnas.0909606106

Finch, C. E., & Rose, M. R. (1995). Hormones and the physiological architecture of life history evolution. *The Quarterly Review of Biology*, *70*(1), 1–52. https://doi.org/10.2307/3037130

Fisher, R. A. (1930). *The Genetical Theory of Natural Selection: A Complete Variorum Edition* (1st ed.). Oxford: Oxford University Press.

Fishilevich, S., Nudel, R., Rappaport, N., Hadar, R., Plaschkes, I., Iny Stein, T., … Cohen, D. (2017). GeneHancer: Genome-wide integration of enhancers and target genes in GeneCards. *Database: The Journal of Biological Databases and Curation*, *2017*. https://doi.org/10.1093/database/bax028

Flatt, T., & Heyland, A. (2011). *Mechanisms of life history evolution: The genetics and physiology of life history traits and trade-offs*. Oxford University Press.

Flinn, M. V., Ponzi, D., & Muehlenbein, M. P. (2012). Hormonal Mechanisms for Regulation of Aggression in Human Coalitions. *Human Nature*, *23*(1), 68–88. https://doi.org/10.1007/s12110-012-9135-y

Folstad, I., & Karter, A. J. (1992). Parasites, Bright Males, and the Immunocompetence Handicap. *The American Naturalist*, *139*(3), 603–622.

Förger, F., & Villiger, P. M. (2020). Immunological adaptations in pregnancy that modulate rheumatoid arthritis disease activity. *Nature Reviews Rheumatology*, *16*(2), 113–122. https://doi.org/10.1038/s41584-019-0351-2

Fortin, J.-P., Triche, T. J., & 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

Fortner, R. T., Sisti, J., Chai, B., Collins, L. C., Rosner, B., Hankinson, S. E., … Eliassen, A. H. (2019). Parity, breastfeeding, and breast cancer risk by hormone receptor status and molecular phenotype: Results from the Nurses' Health Studies. *Breast Cancer Research*, *21*(1), 40. https://doi.org/10.1186/s13058-019-1119-y

Fox, J., & Weisberg, S. (2011). *An R companion to applied Regression* (Second). Thousand Oaks CA: Sage. Retrieved from http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

Fox, M., Berzuini, C., Knapp, L. A., & Glynn, L. M. (2018). Women's Pregnancy Life History and Alzheimer's Risk: Can Immunoregulation Explain the Link? *American Journal of Alzheimer's Disease & Other Dementias®*, *33*(8), 516–526. https://doi.org/10.1177/1533317518786447

Fraga, M. F., Ballestar, E., Paz, M. F., Ropero, S., Setien, F., Ballestar, M. L., … Esteller, M. (2005). Epigenetic differences arise during the lifetime of monozygotic twins. *Proceedings of the National Academy of Sciences*, *102*(30), 10604–10609. https://doi.org/10.1073/pnas.0500398102

Franke, S., & Kulu, H. (2018). Cause-specific mortality by partnership status: Simultaneous analysis using longitudinal data from England and Wales. *Journal*

of Epidemiology and Community Health, *72*(9), 838–844. https://doi.org/10.1136/jech-2017-210339

Franklin, R. E., & Gosling, R. G. (1953). Molecular configuration in sodium thymonucleate. *Nature*, *171*(4356), 740–741.

Frenck, R. W., Blackburn, E. H., & Shannon, K. M. (1998). The rate of telomere sequence loss in human leukocytes varies with age. *Proceedings of the National Academy of Sciences*, *95*(10), 5607–5610.

Fulop, T., Larbi, A., Dupuis, G., Le Page, A., Frost, E. H., Cohen, A. A., … Franceschi, C. (2018). Immunosenescence and Inflamm-Aging As Two Sides of the Same Coin: Friends or Foes? *Frontiers in Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.01960

Gagnon, A., Smith, K. R., Tremblay, M., Vézina, H., Paré, P.-P., & Desjardins, B. (2009). Is there a trade-off between fertility and longevity? A comparative study of women from three large historical databases accounting for mortality selection. *American Journal of Human Biology*, *21*(4), 533–540. https://doi.org/10.1002/ajhb.20893

Gao, X., Colicino, E., Shen, J., Just, A. C., Nwanaji-Enwerem, J. C., Wang, C., … Baccarelli, A. A. (2019). Comparative validation of an epigenetic mortality risk score with three aging biomarkers for predicting mortality risks among older adult males. *International Journal of Epidemiology*, *48*(6), 1958–1971. https://doi.org/10.1093/ije/dyz082

Garagnani, P., Bacalini, M. G., Pirazzini, C., Gori, D., Giuliani, C., Mari, D., … Franceschi, C. (2012). Methylation of ELOVL2 gene as a new epigenetic marker of age. *Aging Cell*, *11*(6), 1132–1134. https://doi.org/10.1111/acel.12005

Garcia, D. N., Saccon, T. D., Pradiee, J., Rincón, J. A. A., Andrade, K. R. S., Rovani, M. T., … Schneider, A. (2019). Effect of caloric restriction and rapamycin on ovarian aging in mice. *GeroScience*, *41*(4), 395–408. https://doi.org/10.1007/s11357-019-00087-x

Geary, D. C. (2015). Evolution of Paternal Investment. In *The Handbook of Evolutionary Psychology*. John Wiley & Sons, Inc. https://doi.org/10.1002/9781119125563.evpsych120

Georgiev, A. V., Kuzawa, C. W., & McDade, T. W. (2016). Early developmental exposures shape trade-offs between acquired and innate immunity in humans. *Evolution, Medicine, and Public Health*, *2016*(1), 256–269. https://doi.org/10.1093/emph/eow022

Germain, C., Meier, A., Jensen, T., Knapnougel, P., Poupon, G., Lazzari, A., … Braud, V. M. (2011). Induction of Lectin-like Transcript 1 (LLT1) Protein Cell Surface Expression by Pathogens and Interferon-γ Contributes to Modulate Immune Responses. *The Journal of Biological Chemistry*, *286*(44), 37964–37975. https://doi.org/10.1074/jbc.M111.285312

Gettler, L. T., McDade, T. W., Feranil, A. B., & Kuzawa, C. W. (2011). Longitudinal evidence that fatherhood decreases testosterone in human males. *Proceedings*

of the National Academy of Sciences, *108*(39), 16194–16199. https://doi.org/10.1073/pnas.1105403108

Gettler, Lee T., Agustin, S. S., & Kuzawa, C. W. (2010). Testosterone, physical activity, and somatic outcomes among Filipino males. *American Journal of Physical Anthropology*, *142*(4), 590–599. https://doi.org/10.1002/ajpa.21282

Gettler, Lee T., Lin, D. T. S., Miegakanda, V., Lew-Levy, S., Eick, G. N., Snodgrass, J. J., … Boyette, A. H. (2020). Epigenetic aging in children from a small-scale farming society in The Congo Basin: Associations with child growth and family conflict. *Developmental Psychobiology*, *62*(2), 138–153. https://doi.org/10.1002/dev.21935

Gettler, Lee T., McDade, T. W., Agustin, S. S., Feranil, A. B., & Kuzawa, C. W. (2013). Do testosterone declines during the transition to marriage and fatherhood relate to men's sexual behavior? Evidence from the Philippines. *Hormones and Behavior*, *64*(5), 755–763. https://doi.org/10.1016/j.yhbeh.2013.08.019

Gettler, Lee T., McDade, T. W., Agustin, S. S., Feranil, A. B., & Kuzawa, C. W. (2014). Testosterone, Immune Function, and Life History Transitions in Filipino Males (Homo sapiens). *International Journal of Primatology*, *35*(3–4), 787–804. https://doi.org/10.1007/s10764-014-9749-5

Ghantous, A., Hernandez-Vargas, H., & Herceg, Z. (2018). DNA Methylation Analysis from Blood Spots: Increasing Yield and Quality for Genome-Wide and Locus-Specific Methylation Analysis. In J. Tost (Ed.), *DNA Methylation Protocols* (pp.

605–619). New York, NY: Springer. https://doi.org/10.1007/978-1-4939-7481-8_31

Gibson, J., Russ, T. C., Clarke, T.-K., Howard, D. M., Hillary, R. F., Evans, K. L., … Marioni, R. E. (2019). A meta-analysis of genome-wide association studies of epigenetic age acceleration. *PLOS Genetics*, 30. https://doi.org/10.1371/journal.pgen.1008104

Gilbert, S. F. (2012). Commentary: 'The Epigenotype' by C.H. Waddington. *International Journal of Epidemiology*, *41*(1), 20–23. https://doi.org/10.1093/ije/dyr186

Gillis, J., Mistry, M., & Pavlidis, P. (2010). Gene function analysis in complex data sets using ErmineJ. *Nature Protocols*, *5*(6), 1148–1159. https://doi.org/10.1038/nprot.2010.78

Gillis, J., & Pavlidis, P. (2011). The Impact of Multifunctional Genes on "Guilt by Association" Analysis. *PLOS ONE*, *6*(2), e17258. https://doi.org/10.1371/journal.pone.0017258

Gluckman, P. D., & Hanson, M. A. (2004). Living with the past: Evolution, development, and patterns of disease. *Science*, *305*(5691), 1733–1736.

Gluckman, P. D., Hanson, M. A., Bateson, P., Beedle, A. S., Law, C. M., Bhutta, Z. A., … others. (2009). Towards a new developmental synthesis: Adaptive developmental plasticity and human disease. *The Lancet*, *373*(9675), 1654–1657.

Glynn, L. M. (2010). Giving birth to a new brain: Hormone exposures of pregnancy influence human memory. *Psychoneuroendocrinology*, *35*(8), 1148–1155. https://doi.org/10.1016/j.psyneuen.2010.01.015

Gokhman, D., Mishol, N., de Manuel, M., de Juan, D., Shuqrun, J., Meshorer, E., … Carmel, L. (2019). Reconstructing Denisovan Anatomy Using DNA Methylation Maps. *Cell*, *179*(1), 180-192.e10. https://doi.org/10.1016/j.cell.2019.08.035

Gokhman, D., Nissim-Rafinia, M., Agranat-Tamir, L., Housman, G., García-Pérez, R., Lizano, E., … Carmel, L. (2020). Differential DNA methylation of vocal and facial anatomy genes in modern humans. *Nature Communications*, *11*(1), 1189. https://doi.org/10.1038/s41467-020-15020-6

Goldman, E. A., Chiou, K. L., Brent, L. J. N., Montague, M. J., Platt, M. L., Horvath, J. E., … Snyder-Mackler, N. (2019). *An epigenetic measure of biological aging in rhesus macaques*. Presented at the The 88th Annual Meeting of the American Association of Physical Anthropologists, Cleveland, Ohio. Retrieved from https://meeting.physanth.org/program/2019/session45/goldman-2019-an-epigenetic-measure-of-biological-aging-in-rhesus-macaques.html

Goldman, E. A., Eick, G. N., Compton, D., Kowal, P., Snodgrass, J. J., Eisenberg, D. T. A., & Sterner, K. N. (2017). Evaluating minimally invasive sample collection methods for telomere length measurement. *American Journal of Human Biology*, e23062-n/a.

Gopalan, S., Carja, O., Fagny, M., Patin, E., Myrick, J. W., McEwen, L. M., … Henn, B. M. (2017). Trends in DNA Methylation with Age Replicate Across Diverse Human

Populations. *Genetics*, *206*(3), 1659–1674. https://doi.org/10.1534/genetics.116.195594

Gorski, D. (2010, June 14). The Human Genome Project: Hype meets reality | ScienceBlogs. Retrieved September 24, 2020, from https://scienceblogs.com/insolence/2010/06/14/the-human-genome-project-hype-meets-real

Gray, P. B., & Anderson, K. G. (2010). *Fatherhood: Evolution and human paternal behavior*. Harvard University Press.

Gray, P. B., Kahlenberg, S. M., Barrett, E. S., Lipson, S. F., & Ellison, P. T. (2002). Marriage and fatherhood are associated with lower testosterone in males. *Evolution and Human Behavior*, *23*(3), 193–201.

Gray, P. B., McHale, T. S., & Carré, J. M. (2017). A review of human male field studies of hormones and behavioral reproductive effort. *Hormones and Behavior*, *91*, 52–67. https://doi.org/10.1016/j.yhbeh.2016.07.004

Gray, R. H., Li, X., Kigozi, G., Serwadda, D., Brahmbhatt, H., Wabwire-Mangen, F., … Quinn, T. C. (2005). Increased risk of incident HIV during pregnancy in Rakai, Uganda: A prospective study. *The Lancet*, *366*(9492), 1182–1188.

Grayson, D. K. (1993). Differential mortality and the Donner Party disaster. *Evolutionary Anthropology: Issues, News, and Reviews*, *2*(5), 151–159. https://doi.org/10.1002/evan.1360020502

Greally, J. M. (2018). A user's guide to the ambiguous word "epigenetics." *Nature Reviews Molecular Cell Biology*, *19*(4), 207–208. https://doi.org/10.1038/nrm.2017.135

Grundy, E. (2009). Women's fertility and mortality in late mid life: A comparison of three contemporary populations. *American Journal of Human Biology*, *21*(4), 541–547. https://doi.org/10.1002/ajhb.20953

Grundy, E., & Kravdal, Ø. (2008). Reproductive History and Mortality in Late Middle Age among Norwegian Men and Women. *American Journal of Epidemiology*, *167*(3), 271–279. https://doi.org/10.1093/aje/kwm295

Grundy, E., & Read, S. (2015). Pathways from fertility history to later life health: Results from analyses of the English Longitudinal Study of Ageing. *Demographic Research*, *32*, 107–146. https://doi.org/10.4054/DemRes.2015.32.4

Grundy, E., & Tomassini, C. (2005). Fertility history and health in later life: A record linkage study in England and Wales. *Social Science & Medicine*, *61*(1), 217–228. https://doi.org/10.1016/j.socscimed.2004.11.046

Grunspan, D. Z., Nesse, R. M., Barnes, M. E., & Brownell, S. E. (2018). Core principles of evolutionary medicine: A Delphi study. *Evolution, Medicine, and Public Health*, *2018*(1), 13–23. https://doi.org/10.1093/emph/eox025

Guan, H.-B., Wu, Q.-J., & Gong, T.-T. (2013). Parity and Kidney Cancer Risk: Evidence from Epidemiologic Studies. *Cancer Epidemiology and Prevention Biomarkers*, *22*(12), 2345–2353. https://doi.org/10.1158/1055-9965.EPI-13-0759-T

Gupta, S., Kim, S. Y., Artis, S., Molfese, D. L., Schumacher, A., Sweatt, J. D., … Lubin, F. D. (2010). Histone Methylation Regulates Memory Formation. *The Journal of Neuroscience*, *30*(10), 3589–3599. https://doi.org/10.1523/JNEUROSCI.3732-09.2010

Gurdon, J. B. (1962). Adult frogs derived from the nuclei of single somatic cells. *Developmental Biology*, *4*(2), 256–273. https://doi.org/10.1016/0012-1606(62)90043-X

Gurdon, J. B., Elsdale, T. R., & Fischberg, M. (1958). Sexually Mature Individuals of Xenopus laevis from the Transplantation of Single Somatic Nuclei. *Nature*, *182*(4627), 64–65. https://doi.org/10.1038/182064a0

Gurven, M., Costa, M., Ben Trumble, Stieglitz, J., Beheim, B., Eid Rodriguez, D., … Kaplan, H. (2016). Health costs of reproduction are minimal despite high fertility, mortality and subsistence lifestyle. *Scientific Reports*, *6*, 30056. https://doi.org/10.1038/srep30056

Gurven, M. D., Trumble, B. C., Stieglitz, J., Yetish, G., Cummings, D., Blackwell, A. D., … Pontzer, H. (2016). High resting metabolic rate among Amazonian forager-horticulturalists experiencing high pathogen burden. *American Journal of Physical Anthropology*, n/a-n/a. https://doi.org/10.1002/ajpa.23040

Haig, D. (2004). The (dual) origin of epigenetics. *Cold Spring Harbor Symposia on Quantitative Biology*, *69*, 67–70. Cold Spring Harbor Laboratory Press. Retrieved from http://symposium.cshlp.org/content/69/67.short

Haldane, J. B. S. (1941). New paths in genetics. *New Paths in Genetics.*

Hamilton, W.D. (1966). The moulding of senescence by natural selection. *Journal of Theoretical Biology*, *12*(1), 12–45. https://doi.org/10.1016/0022-5193(66)90184-6

Hamilton, William D. (1964). The genetical evolution of social behaviour. II. *Journal of Theoretical Biology*, *7*(1), 17–52.

Hamilton, William D, & Zuk, M. (1982). Heritable true fitness and bright birds: A role for parasites? *Science*, *218*(4570), 384–387.

Hannum, G., Guinney, J., Zhao, L., Zhang, L., Hughes, G., Sadda, S., … Zhang, K. (2013). Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates. *Molecular Cell*, *49*(2), 359–367. https://doi.org/10.1016/j.molcel.2012.10.016

Hansen, K. (2015). IlluminaHumanMethylation450kanno. Ilmn12. Hg19: Annotation for illumina's 450k methylation arrays. *R Package, Version 0.2*, *1*.

Harley, C. B., Futcher, A. B., & Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. *Nature*, *345*(6274), 458–460.

Harshman, L. G., & Zera, A. J. (2007). The cost of reproduction: The devil in the details. *Trends in Ecology & Evolution*, *22*(2), 80–86. https://doi.org/10.1016/j.tree.2006.10.008

Haycock, P. C., Heydon, E. E., Kaptoge, S., Butterworth, A. S., Thompson, A., & Willeit, P. (2014). Leucocyte telomere length and risk of cardiovascular disease: Systematic review and meta-analysis. *BMJ*, *349*, g4227.

Heckwolf, M. J., Meyer, B. S., Häsler, R., Höppner, M. P., Eizaguirre, C., & Reusch, T. B. H. (2020). Two different epigenetic information channels in wild three-spined

sticklebacks are involved in salinity adaptation. *Science Advances*, *6*(12), eaaz1138. https://doi.org/10.1126/sciadv.aaz1138

Heilbronn, L. K., & Ravussin, E. (2003). Calorie restriction and aging: Review of the literature and implications for studies in humans. *The American Journal of Clinical Nutrition*, *78*(3), 361–369. https://doi.org/10.1093/ajcn/78.3.361

Helle, S., Lummaa, V., & Jokela, J. (2004). Accelerated immunosenescence in preindustrial twin mothers. *Proceedings of the National Academy of Sciences*, *101*(33), 12391–12396. https://doi.org/10.1073/pnas.0402215101

Helle, Samuli. (2017). Accounting for measurement error in human life history trade-offs using structural equation modeling. *American Journal of Human Biology*, e23075. https://doi.org/10.1002/ajhb.23075

Helle, Samuli. (2019). Search for a Resource-Based Trade-off Between Lifetime Reproductive Effort and Women's Postreproductive Survival in Preindustrial Sweden. *The Journals of Gerontology: Series A*, *74*(5), 642–647. https://doi.org/10.1093/gerona/gly203

Henze, L., Schwinge, D., & Schramm, C. (2020). The Effects of Androgens on T Cells: Clues to Female Predominance in Autoimmune Liver Diseases? *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.01567

Heo, J. B., & Sung, S. (2011). Vernalization-Mediated Epigenetic Silencing by a Long Intronic Noncoding RNA. *Science*, *331*(6013), 76–79. https://doi.org/10.1126/science.1197349

Hill, K., & Kaplan, and H. (1999). Life History Traits in Humans: Theory and Empirical Studies. *Annual Review of Anthropology*, *28*(1), 397–430. https://doi.org/10.1146/annurev.anthro.28.1.397

Hill, K. R., & Hurtado, A. M. (1996). *Ache Life History: The Ecology and Demography of a Foraging People*. Transaction Publishers.

Hillary, R. F., Stevenson, A. J., McCartney, D. L., Campbell, A., Walker, R. M., Howard, D. M., … Marioni, R. E. (2020). Epigenetic measures of ageing predict the prevalence and incidence of leading causes of death and disease burden. *Clinical Epigenetics*, *12*(1), 115. https://doi.org/10.1186/s13148-020-00905-6

Hinde, K., & Milligan, L. A. (2011). Primate milk: Proximate mechanisms and ultimate perspectives. *Evolutionary Anthropology: Issues, News, and Reviews*, *20*(1), 9–23. https://doi.org/10.1002/evan.20289

Hlavac, M. (2014). *stargazer: LaTeX code and ASCII text for well-formatted regression and summary statistics tables*.

Hoekzema, E., Barba-Müller, E., Pozzobon, C., Picado, M., Lucco, F., García-García, D., … Vilarroya, O. (2017). Pregnancy leads to long-lasting changes in human brain structure. *Nature Neuroscience*, *20*(2), 287–296. https://doi.org/10.1038/nn.4458

Horvath, S. (2013). DNA methylation age of human tissues and cell types. *Genome Biology*, *14*, 3156. https://doi.org/10.1186/gb-2013-14-10-r115

Horvath, S., Erhart, W., Brosch, M., Ammerpohl, O., Schönfels, W. von, Ahrens, M., … Hampe, J. (2014). Obesity accelerates epigenetic aging of human liver.

Proceedings of the National Academy of Sciences, *111*(43), 15538–15543. https://doi.org/10.1073/pnas.1412759111

Horvath, S., Gurven, M., Levine, M. E., Trumble, B. C., Kaplan, H., Allayee, H., … Assimes, T. L. (2016). An epigenetic clock analysis of race/ethnicity, sex, and coronary heart disease. *Genome Biology*, *17*. https://doi.org/10.1186/s13059-016-1030-0

Horvath, S., & Levine, A. J. (2015). HIV-1 Infection Accelerates Age According to the Epigenetic Clock. *Journal of Infectious Diseases*, *212*(10), 1563–1573. https://doi.org/10.1093/infdis/jiv277

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

Hoshino, A., Horvath, S., Sridhar, A., Chitsazan, A., & Reh, T. A. (2019). Synchrony and asynchrony between an epigenetic clock and developmental timing. *Scientific Reports*, *9*(1), 1–12. https://doi.org/10.1038/s41598-019-39919-3

Hosseini, S. M., Talaei-khozani, T., Sani, M., & Owrangi, B. (2014). Differentiation of Human Breast-Milk Stem Cells to Neural Stem Cells and Neurons. *Neurology Research International*, *2014*, 1–8. https://doi.org/10.1155/2014/807896

Houseman, E. A., Accomando, W. P., Koestler, D. C., Christensen, B. C., Marsit, C. J., Nelson, H. H., … Kelsey, K. T. (2012a). DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics*, *13*(1), 1–16. https://doi.org/10.1186/1471-2105-13-86

Houseman, E. A., Accomando, W. P., Koestler, D. C., Christensen, B. C., Marsit, C. J., Nelson, H. H., … Kelsey, K. T. (2012b). DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics*, *13*(1), 86. https://doi.org/10.1186/1471-2105-13-86

Houseman, E. A., Molitor, J., & Marsit, C. J. (2014). Reference-free cell mixture adjustments in analysis of DNA methylation data. *Bioinformatics*, *30*(10), 1431–1439. https://doi.org/10.1093/bioinformatics/btu029

Hu, Y., & Goldman, N. (1990). Mortality Differentials by Marital Status: An International Comparison. *Demography*, *27*(2), 233–250. https://doi.org/10.2307/2061451

Huang, R.-C., Lillycrop, K. A., Beilin, L. J., Godfrey, K. M., Anderson, D., Mori, T. A., … Melton, P. E. (2019). Epigenetic Age Acceleration in Adolescence Associates With BMI, Inflammation, and Risk Score for Middle Age Cardiovascular Disease. *The Journal of Clinical Endocrinology & Metabolism*, *104*(7), 3012–3024. https://doi.org/10.1210/jc.2018-02076

Huo, D., Feng, Y., Haddad, S., Zheng, Y., Yao, S., Han, Y.-J., … Haiman, C. A. (2016). Genome-wide association studies in women of African ancestry identified 3q26.21 as a novel susceptibility locus for oestrogen receptor negative breast cancer. *Human Molecular Genetics*, *25*(21), 4835–4846. https://doi.org/10.1093/hmg/ddw305

Hurt, L. S., Ronsmans, C., & Thomas, S. L. (2006). The effect of number of births on women's mortality: Systematic review of the evidence for women who have

completed their childbearing. *Population Studies*, *60*(1), 55–71. https://doi.org/10.1080/00324720500436011

James, G., Witten, D., Hastie, T., & Tibshirani, R. (Eds.). (2013). *An introduction to statistical learning: With applications in R*. New York: Springer.

Jasienska, G. (2009). Reproduction and lifespan: Trade-offs, overall energy budgets, intergenerational costs, and costs neglected by research. *American Journal of Human Biology*, *21*(4), 524–532. https://doi.org/10.1002/ajhb.20931

Jasienska, G., Bribiescas, R. G., Furberg, A.-S., Helle, S., & Núñez-de la Mora, A. (2017). Human reproduction and health: An evolutionary perspective. *The Lancet*, *390*(10093), 510–520.

Jirtle, R. L., & Tyson, F. L. (Eds.). (2013). *Environmental Epigenomics in Health and Disease*. Berlin, Heidelberg: Springer Berlin Heidelberg. Retrieved from http://link.springer.com/10.1007/978-3-642-36827-1

Johannsen, W. (1911). The Genotype Conception of Heredity. *The American Naturalist*, *45*(531), 129–159.

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

Jones, M. J., Islam, S. A., Edgar, R. D., & Kobor, M. S. (2015). Adjusting for Cell Type Composition in DNA Methylation Data Using a Regression-Based Approach. In P. Haggarty & K. Harrison (Eds.), *Population Epigenetics* (Vol. 1589, pp. 99–106). New York, NY: Springer New York. https://doi.org/10.1007/7651_2015_262

Joo, J. E., Wong, E. M., Baglietto, L., Jung, C.-H., Tsimiklis, H., Park, D. J., … Southey, M. C. (2013). The use of DNA from archival dried blood spots with the Infinium HumanMethylation450 array. *BMC Biotechnology*, *13*, 23. https://doi.org/10.1186/1472-6750-13-23

Jovanovic, T., Vance, L. A., Cross, D., Knight, A. K., Kilaru, V., Michopoulos, V., … Smith, A. K. (2017). Exposure to Violence Accelerates Epigenetic Aging in Children. *Scientific Reports*, *7*(1), 8962. https://doi.org/10.1038/s41598-017-09235-9

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

Kahn, H. A., Kahn, H. A., & Sempos, C. T. (1989). *Statistical Methods in Epidemiology*. New York, NY: Oxford University Press.

Kamb, S. (2013, October 3). Xconomy: The Human Genome Project Wasn't Overhyped. The Payoff Just Took Time. Retrieved September 24, 2020, from Xconomy website: https://xconomy.com/national/2013/10/03/human-genome-project-wasnt-overhyped-payoff-just-took-time/

Kanai, Y., & Arai, E. (2012). DNA Methylation Alterations in Human Cancers. In *Epigenetics in Human Disease* (pp. 29–52). Elsevier. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/B9780123884152000032

Kananen, L., Nevalainen, T., Jylhävä, J., Marttila, S., Hervonen, A., Jylhä, M., & Hurme, M. (2015). Cytomegalovirus infection accelerates epigenetic aging. *Experimental Gerontology*, *72*, 227–229. https://doi.org/10.1016/j.exger.2015.10.008

Kazemi-Esfarjani, P., Trifiro, M. A., & Pinsky, L. (1995). Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: Possible pathogenetic relevance for the (CAG)n-expanded neuronopathies. *Human Molecular Genetics*, *4*(4), 523–527. https://doi.org/10.1093/hmg/4.4.523

Keizer, R., Dykstra, P. A., & J. Van Lenthe, F. (2012). Parity and men's mortality risks. *European Journal of Public Health*, *22*(3), 343–347. https://doi.org/10.1093/eurpub/ckr024

Keller, E. F. (2000). *The century of the gene*. Cambridge, Mass: Harvard University Press.

Keller, E. F. (2014). From gene action to reactive genomes. *The Journal of Physiology*, *592*(11), 2423–2429. https://doi.org/10.1113/jphysiol.2014.270991

Khashan, A. S., Kenny, L. C., Laursen, T. M., Mahmood, U., Mortensen, P. B., Henriksen, T. B., & O'Donoghue, K. (2011). Pregnancy and the Risk of Autoimmune Disease. *PLOS ONE*, *6*(5), e19658. https://doi.org/10.1371/journal.pone.0019658

Kichaev, G., Bhatia, G., Loh, P.-R., Gazal, S., Burch, K., Freund, M. K., … Price, A. L. (2019). Leveraging Polygenic Functional Enrichment to Improve GWAS Power. *The American Journal of Human Genetics*, *104*(1), 65–75. https://doi.org/10.1016/j.ajhg.2018.11.008

Kimura, M., Hjelmborg, J. V., Gardner, J. P., Bathum, L., Brimacombe, M., Lu, X., … Christensen, K. (2008). Telomere length and mortality: A study of leukocytes in elderly Danish twins. *Am J Epidemiol*, *167*(7), 799–806.

Kirkwood, T. B. L. (1977). Evolution of ageing. *Nature*, *270*(5635), 301–304.

Kirkwood, T. B. L., & Holliday, R. (1979). The evolution of ageing and longevity. *Proceedings of the Royal Society of London. Series B. Biological Sciences*, *205*(1161), 531–546. https://doi.org/10.1098/rspb.1979.0083

Kirkwood, T. B. L., & Rose, M. R. (1991). Evolution of Senescence: Late Survival Sacrificed for Reproduction. *Philosophical Transactions: Biological Sciences*, *332*(1262), 15–24.

Kleiber, C., & Zeileis, A. (2008). *Applied econometrics with R*. Springer Verlag.

Klimek, M., Galbarczyk, A., Nenko, I., Alvarado, L. C., & Jasienska, G. (2014). Digit ratio (2D:4D) as an indicator of body size, testosterone concentration and number of children in human males. *Annals of Human Biology*, *41*(6), 518–523. https://doi.org/10.3109/03014460.2014.902993

Knight, A. K., Craig, J. M., Theda, C., Bækvad-Hansen, M., Bybjerg-Grauholm, J., Hansen, C. S., … Smith, A. K. (2016). An epigenetic clock for gestational age at birth based on blood methylation data. *Genome Biology*, *17*(1). https://doi.org/10.1186/s13059-016-1068-z

Komori, H. K., LaMere, S. A., Hart, T., Head, S. R., Torkamani, A., & Salomon, D. R. (2018). Microdroplet PCR for Highly Multiplexed Targeted Bisulfite Sequencing. In J. Tost (Ed.), *DNA Methylation Protocols* (pp. 333–348). New York, NY: Springer.

Kraus, T. A., Engel, S. M., Sperling, R. S., Kellerman, L., Lo, Y., Wallenstein, S., … Moran, T. M. (2012). Characterizing the Pregnancy Immune Phenotype: Results

of the Viral Immunity and Pregnancy (VIP) Study. *Journal of Clinical Immunology*, *32*(2), 300–311. https://doi.org/10.1007/s10875-011-9627-2

Krebs, R. A., & Loeschcke, V. (1994). Costs and Benefits of Activation of the Heat-Shock Response in Drosophila melanogaster. *Functional Ecology*, *8*(6), 730. https://doi.org/10.2307/2390232

Kresovich, J. K., Harmon, Q. E., Xu, Z., Nichols, H. B., Sandler, D. P., & Taylor, J. A. (2019). Reproduction, DNA methylation and biological age. *Human Reproduction*, *34*(10), 1965–1973. https://doi.org/10.1093/humrep/dez149

Kresovich, J. K., Xu, Z., O'Brien, K. M., Weinberg, C. R., Sandler, D. P., & Taylor, J. A. (2019). Epigenetic mortality predictors and incidence of breast cancer. *Aging*, *11*(24), 11975–11987. https://doi.org/10.18632/aging.102523

Kruger, D. J., & Nesse, R. M. (2006). An evolutionary life-history framework for understanding sex differences in human mortality rates. *Human Nature*, *17*(1), 74–97. https://doi.org/10.1007/s12110-006-1021-z

Kuzawa, C. W. (2005). Fetal origins of developmental plasticity: Are fetal cues reliable predictors of future nutritional environments? *American Journal of Human Biology*, *17*(1), 5–21. https://doi.org/10.1002/ajhb.20091

Kuzawa, C. W. (2007). Developmental origins of life history: Growth, productivity, and reproduction. *American Journal of Human Biology*, *19*(5), 654–661. https://doi.org/10.1002/ajhb.20659

Kuzawa, C. W., Adair, L., Bechayda, S. A., Borja, J. R. B., Carba, D. B., Duazo, P. L., … McDade, T. W. (2020). Evolutionary life history theory as an organising

framework for cohort studies: Insights from the Cebu Longitudinal Health and Nutrition Survey. *Annals of Human Biology*, *47*(2), 94–105. https://doi.org/10.1080/03014460.2020.1742787

Kuzawa, C. W., Adair, L. S., Borja, J., & Mcdade, T. W. (2013). C-reactive protein by pregnancy and lactational status among Filipino young adult women. *American Journal of Human Biology*, *25*(1), 131–134. https://doi.org/10.1002/ajhb.22351

Kuzawa, C. W., & Bragg, J. M. (2012). Plasticity in Human Life History Strategy: Implications for Contemporary Human Variation and the Evolution of Genus *Homo*. *Current Anthropology*, *53*(S6), S369–S382. https://doi.org/10.1086/667410

Kuzawa, C. W., Fried, R., Borja, J., & McDade, T. (2017). Maternal pregnancy C-reactive protein predicts offspring birth size and body composition in metropolitan Cebu, Philippines. *Journal of Developmental Origins of Health and Disease*, *8*(6), 674–681.

Kuzawa, C. W., Georgiev, A. V., McDade, T. W., Bechayda, S. A., & Gettler, L. T. (2016). Is There a Testosterone Awakening Response in Humans? *Adaptive Human Behavior and Physiology*, *2*(2), 166–183. https://doi.org/10.1007/s40750-015-0038-0

Kuzawa, C. W., & Sweet, E. (2009). Epigenetics and the embodiment of race: Developmental origins of US racial disparities in cardiovascular health. *American Journal of Human Biology*, *21*(1), 2–15. https://doi.org/10.1002/ajhb.20822

Kuzawa, C. W., & Thayer, Z. M. (2011). Timescales of human adaptation: The role of epigenetic processes. *Epigenomics*, *3*(2), 221–234. https://doi.org/10.2217/epi.11.11

La Rocca, C., Carbone, F., Longobardi, S., & Matarese, G. (2014). The immunology of pregnancy: Regulatory T cells control maternal immune tolerance toward the fetus. *Immunology Letters*, *162*(1, Part A), 41–48. https://doi.org/10.1016/j.imlet.2014.06.013

Lanciers, S., Despinasse, B., Mehta, D. I., & Blecker, U. (1999). Increased susceptibility to Helicobacter pylori infection in pregnancy. *Infect Dis Obstet Gynecol*, *7*(4), 195–198.

Lander, E. S. (2015). Brave new genome. *New England Journal of Medicine*, *373*(1), 5–8.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., … Morgan, M. J. (2001). Initial sequencing and analysis of the human genome. *Nature*, *409*(6822), 860–921. https://doi.org/10.1038/35057062

Lane-Cordova, A. D., Puterman, E., Gunderson, E. P., Chan, C., Hou, L., & Carnethon, M. (2017). Gravidity is not associated with telomere length in a biracial cohort of middle-aged women: The Coronary Artery Risk Development in Young Adults (CARDIA) study. *PLOS ONE*, *12*(10), e0186495. https://doi.org/10.1371/journal.pone.0186495

Lappalainen, T., & Greally, J. M. (2017). Associating cellular epigenetic models with human phenotypes. *Nature Reviews Genetics*. Retrieved from https://www.nature.com/nrg/journal/vaop/ncurrent/full/nrg.2017.32.html

Le Bourg, É. (2007). Does reproduction decrease longevity in human beings? *Ageing Research Reviews*, *6*(2), 141–149. https://doi.org/10.1016/j.arr.2007.04.002

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.

Levine, M. E. (2013). Modeling the Rate of Senescence: Can Estimated Biological Age Predict Mortality More Accurately Than Chronological Age? *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *68*(6), 667–674. https://doi.org/10.1093/gerona/gls233

Levine, M. E., Hosgood, H. D., Chen, B., Absher, D., Assimes, T., & Horvath, S. (2015). DNA methylation age of blood predicts future onset of lung cancer in the women's health initiative. *Aging*, *7*(9), 690–700. https://doi.org/10.18632/aging.100809

Levine, M. E., Lu, A. T., Bennett, D. A., & Horvath, S. (2015). Epigenetic age of the pre-frontal cortex is associated with neuritic plaques, amyloid load, and Alzheimer's disease related cognitive functioning. *Aging*, *7*(12), 1198–1211. https://doi.org/10.18632/aging.100864

Levine, M. E., Lu, A. T., Chen, B. H., Hernandez, D. G., Singleton, A. B., Ferrucci, L., … Horvath, S. (2016). Menopause accelerates biological aging. *Proceedings of the*

National Academy of Sciences, 201604558. https://doi.org/10.1073/pnas.1604558113

Levine, M. E., Lu, A. T., Quach, A., Chen, B. H., Assimes, T. L., Hou, L., … Ferrucci, L. (2018). An epigenetic biomarker of aging for lifespan and healthspan. *Aging*, *10*, 19.

Li, W., Ruan, W., Lu, Z., & Wang, D. (2019). Parity and risk of maternal cardiovascular disease: A dose–response meta-analysis of cohort studies. *European Journal of Preventive Cardiology*, *26*(6), 592–602. https://doi.org/10.1177/2047487318818265

Li, X., Ploner, A., Wang, Y., Magnusson, P. K., Reynolds, C., Finkel, D., … Hägg, S. (2020). Longitudinal trajectories, correlations and mortality associations of nine biological ages across 20-years follow-up. *ELife*, *9*, e51507. https://doi.org/10.7554/eLife.51507

Lin, Q., Weidner, C. I., Costa, I. G., Marioni, R. E., Ferreira, M. R. P., Deary, I. J., & Wagner, W. (2016). DNA methylation levels at individual age-associated CpG sites can be indicative for life expectancy. *Aging (Albany NY)*, *8*(2), 394–401.

Lira-Medeiros, C. F., Parisod, C., Fernandes, R. A., Mata, C. S., Cardoso, M. A., & Ferreira, P. C. G. (2010). Epigenetic Variation in Mangrove Plants Occurring in Contrasting Natural Environment. *PLoS ONE*, *5*(4), e10326. https://doi.org/10.1371/journal.pone.0010326

Liu, C., Marioni, R. E., Hedman, Å. K., Pfeiffer, L., Tsai, P.-C., Reynolds, L. M., … Levy, D. (2018). A DNA methylation biomarker of alcohol consumption. *Molecular Psychiatry*, *23*(2), 422–433. https://doi.org/10.1038/mp.2016.192

Liu, J., Qiao, X., Qian, W., Hou, X., Hayes, J., & Chen, J. (2004). Motilin in human milk and its elevated plasma concentration in lactating women. *Journal of Gastroenterology and Hepatology*, *19*(10), 1187–1191. https://doi.org/10.1111/j.1440-1746.2004.03458.x

Liu, Z., Chen, B. H., Assimes, T. L., Ferrucci, L., Horvath, S., & Levine, M. E. (2019). The role of epigenetic aging in education and racial/ethnic mortality disparities among older U.S. Women. *Psychoneuroendocrinology*, *104*, 18–24. https://doi.org/10.1016/j.psyneuen.2019.01.028

Logue, M. W., Smith, A. K., Wolf, E. J., Maniates, H., Stone, A., Schichman, S. A., … Miller, M. W. (2017). The correlation of methylation levels measured using Illumina 450K and EPIC BeadChips in blood samples. *Epigenomics*, *9*(11), 1363–1371. https://doi.org/10.2217/epi-2017-0078

Lopes, R., Korkmaz, G., Revilla, S. A., van Vliet, R., Nagel, R., Custers, L., … Agami, R. (2018). CUEDC1 is a primary target of ERα essential for the growth of breast cancer cells. *Cancer Letters*, *436*, 87–95. https://doi.org/10.1016/j.canlet.2018.08.018

Lotta, L. A., Gulati, P., Day, F. R., Payne, F., Ongen, H., van de Bunt, M., … Cambridge FPLD1 Consortium. (2017). Integrative genomic analysis implicates limited

peripheral adipose storage capacity in the pathogenesis of human insulin resistance. *Nature Genetics*, *49*(1), 17–26. https://doi.org/10.1038/ng.3714

Lowe, D., Horvath, S., & Raj, K. (2016). Epigenetic clock analyses of cellular senescence and ageing. *Oncotarget*, *7*(8), 8524.

Lu, A. T., Quach, A., Wilson, J. G., Reiner, A. P., Aviv, A., Raj, K., … Horvath, S. (2019). DNA methylation GrimAge strongly predicts lifespan and healthspan. *Aging*, *11*(2), 303–327. https://doi.org/10.18632/aging.101684

Lu, A. T., Seeboth, A., Tsai, P.-C., Sun, D., Quach, A., Reiner, A. P., … Horvath, S. (2019). DNA methylation-based estimator of telomere length. *Aging*. https://doi.org/10.18632/aging.102173

Lu, A. T., Xue, L., Salfati, E. L., Chen, B. H., Ferrucci, L., Levy, D., … Horvath, S. (2018). GWAS of epigenetic aging rates in blood reveals a critical role for TERT. *Nature Communications*, *9*(1), 387. https://doi.org/10.1038/s41467-017-02697-5

Luetjens, C. M., & Weinbauer, G. F. (2012). Testosterone: Biosynthesis, transport, metabolism and (non-genomic) actions. In Eberhard Nieschlag, H. M. Behre, & S. Nieschlag (Eds.), *Testosterone: Action, deficiency, substitution* (4ed., pp. 15–32). Cambridge, UK: Cambridge University Press.

Lurie, S., Rahamim, E., Piper, I., Golan, A., & Sadan, O. (2008). Total and differential leukocyte counts percentiles in normal pregnancy. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *136*(1), 16–19.

Lv, H., Wu, H., Yin, J., Qian, J., & Ge, J. (2015). Parity and Cardiovascular Disease Mortality: A Dose-Response Meta-Analysis of Cohort Studies. *Scientific Reports*, *5*(1), 13411. https://doi.org/10.1038/srep13411

Lycett, J. E., Dunbar, R. I. M., & Voland, E. (2000). Longevity and the costs of reproduction in a historical human population. *Proceedings of the Royal Society of London B: Biological Sciences*, *267*(1438), 31–35.

Maglott, D., Ostell, J., Pruitt, K. D., & Tatusova, T. (2007). Entrez Gene: Gene-centered information at NCBI. *Nucleic Acids Research*, *35*(suppl_1), D26–D31.

Maksimovic, J., Gordon, L., & Oshlack, A. (2012). SWAN: Subset-quantile Within Array Normalization for Illumina Infinium HumanMethylation450 BeadChips. *Genome Biology*, *13*(6), R44. https://doi.org/10.1186/gb-2012-13-6-r44

Manini, T. M. (2010). Energy Expenditure and Aging. *Ageing Research Reviews*, *9*(1), 1. https://doi.org/10.1016/j.arr.2009.08.002

Marini, S., Davis, K. A., Soare, T. W., Zhu, Y., Suderman, M. J., Simpkin, A. J., … Dunn, E. C. (2020). Adversity exposure during sensitive periods predicts accelerated epigenetic aging in children. *Psychoneuroendocrinology*, *113*, 104484. https://doi.org/10.1016/j.psyneuen.2019.104484

Marioni, R. E., Harris, S. E., Shah, S., McRae, A. F., Zglinicki, T. von, Martin-Ruiz, C., … Deary, I. J. (2016). The epigenetic clock and telomere length are independently associated with chronological age and mortality. *International Journal of Epidemiology*, dyw041. https://doi.org/10.1093/ije/dyw041

Marioni, R. E., Shah, S., McRae, A. F., Chen, B. H., Colicino, E., Harris, S. E., … Deary, I. J. (2015). DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biology*, *16*(1), 25. https://doi.org/10.1186/s13059-015-0584-6

Marioni, R. E., Shah, S., McRae, A. F., Ritchie, S. J., Muniz-Terrera, G., Harris, S. E., … Deary, I. J. (2015). The epigenetic clock is correlated with physical and cognitive fitness in the Lothian Birth Cohort 1936. *International Journal of Epidemiology*, *44*(4), 1388–1396. https://doi.org/10.1093/ije/dyu277

Marks, J. (2012). The Origins of Anthropological Genetics. *Current Anthropology*, *53*(S5), S161–S172. JSTOR. https://doi.org/10.1086/662333

Marshall, K. E., & Sinclair, B. J. (2010). Repeated stress exposure results in a survival-reproduction trade-off in Drosophila melanogaster. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1683), 963–969. https://doi.org/10.1098/rspb.2009.1807

Maynard Smith, J. (1958). The Effects of Temperature and of Egg-Laying on the Longevity of Drosophila Subobscura. *Journal of Experimental Biology*, *35*(4), 832–842.

McClintock, B. (1950). The origin and behavior of mutable loci in maize. *Proceedings of the National Academy of Sciences*, *36*(6), 344–355. https://doi.org/10.1073/pnas.36.6.344

McClintock, B. (1984). The significance of responses of the genome to challenge. *Science*, *226*(4676), 792–801. https://doi.org/10.1126/science.15739260

McCombe, P. A. (2018). The Short and Long-Term Effects of Pregnancy on Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis. *Journal of Clinical Medicine*, *7*(12), 494. https://doi.org/10.3390/jcm7120494

McDade, Thomas W. (2014). Development and validation of assay protocols for use with dried blood spot samples: T.W. McDade. *American Journal of Human Biology*, *26*(1), 1–9. https://doi.org/10.1002/ajhb.22463

McDade, Thomas W., Borja, J. B., Largado, F., Adair, L. S., & Kuzawa, C. W. (2016). Adiposity and Chronic Inflammation in Young Women Predict Inflammation during Normal Pregnancy in the Philippines. *The Journal of Nutrition*, *146*(2), 353–357. https://doi.org/10.3945/jn.115.224279

McDade, Thomas W., Georgiev, A. V., & Kuzawa, C. W. (2016). Trade-offs between acquired and innate immune defenses in humans. *Evolution, Medicine, and Public Health*, *2016*(1), 1–16. https://doi.org/10.1093/emph/eov033

McDade, Thomas W., Ryan, C., Jones, M. J., MacIsaac, J. L., Morin, A. M., Meyer, J. M., … Kuzawa, C. W. (2017). Social and physical environments early in development predict DNA methylation of inflammatory genes in young adulthood. *Proceedings of the National Academy of Sciences*, *114*(29), 7611–7616. https://doi.org/10.1073/pnas.1620661114

McDade, Thomas W., Ryan, C. P., Jones, M. J., Hoke, M. K., Borja, J., Miller, G. E., … Kobor, M. S. (2019). Genome-wide analysis of DNA methylation in relation to socioeconomic status during development and early adulthood. *American*

Journal of Physical Anthropology, *169*(1), 3–11. https://doi.org/10.1002/ajpa.23800

McDade, T.W., Reyes-García, V., Tanner, S., Huanca, T., & Leonard, W. R. (2008). Maintenance versus growth: Investigating the costs of immune activation among children in lowland Bolivia. *American Journal of Physical Anthropology*, *136*(4), 478–484. https://doi.org/10.1002/ajpa.20831

McEwen, L. M., Goodman, S. J., Kobor, M. S., & Jones, M. J. (2017). The DNA methylome: An interface between the environment, immunity, and ageing. In *The Ageing Immune System and Health* (pp. 35–52). Springer, Cham. https://doi.org/10.1007/978-3-319-43365-3_3

McEwen, L. M., Morin, A. M., Edgar, R. D., MacIsaac, J. L., Jones, M. J., Dow, W. H., … Rehkopf, D. H. (2017). Differential DNA methylation and lymphocyte proportions in a Costa Rican high longevity region. *Epigenetics & Chromatin*, *10*(1), 1–14. https://doi.org/10.1186/s13072-017-0128-2

McEwen, L. M., O'Donnell, K. J., McGill, M. G., Edgar, R. D., Jones, M. J., MacIsaac, J. L., … Kobor, M. S. (2019). The PedBE clock accurately estimates DNA methylation age in pediatric buccal cells. *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.1820843116

McGowan, P. O., Sasaki, A., D'Alessio, A. C., Dymov, S., Labonté, B., Szyf, M., … Meaney, M. J. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nature Neuroscience*, *12*(3), 342–348. https://doi.org/10.1038/nn.2270

McGrath, J., & Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*, *37*(1), 179–183. https://doi.org/10.1016/0092-8674(84)90313-1

McNeilly, A. S., Tay, C. C. K., & Glasier, A. (1994). Physiological mechanisms underlying lactational amenorrhea. *Annals of the New York Academy of Sciences*, *709*(1), 145–155. https://doi.org/10.1111/j.1749-6632.1994.tb30394.x

Meaney, M. J. (2001). Maternal Care, Gene Expression, and the Transmission of Individual Differences in Stress Reactivity Across Generations. *Annual Review of Neuroscience*, *24*(1), 1161–1192. https://doi.org/10.1146/annurev.neuro.24.1.1161

Meaney, M. J. (2010). Epigenetics and the biological definition of gene x environment interactions. *Child Development*, *81*(1), 41–79.

Mehta, D., Bruenig, D., Lawford, B., Harvey, W., Carrillo-Roa, T., Morris, C. P., … Voisey, J. (2018). Accelerated DNA methylation aging and increased resilience in veterans: The biological cost for soldiering on. *Neurobiology of Stress*, *8*, 112–119. https://doi.org/10.1016/j.ynstr.2018.04.001

Mendel, G. (1866). Versuche über Pflanzenhybriden. *Verhandlungen Des Naturforschenden Vereines in Brunn 4: 3*, *44*.

Mendizabal, I., Shi, L., Keller, T. E., Konopka, G., Preuss, T. M., Hsieh, T.-F., … Yi, S. V. (2016). Comparative Methylome Analyses Identify Epigenetic Regulatory Loci of Human Brain Evolution. *Molecular Biology and Evolution*, *33*(11), 2947–2959. https://doi.org/10.1093/molbev/msw176

Merico, D., Isserlin, R., Stueker, O., Emili, A., & Bader, G. D. (2010). Enrichment Map: A Network-Based Method for Gene-Set Enrichment Visualization and Interpretation. *PLOS ONE*, *5*(11), e13984. https://doi.org/10.1371/journal.pone.0013984

Meyne, J., Ratliff, R. L., & Moyzis, R. K. (1989). Conservation of the human telomere sequence (TTAGGG)n among vertebrates. *Proceedings of the National Academy of Sciences*, *86*(18), 7049–7053.

Michels, K. B. (Ed.). (2012). *Epigenetic epidemiology*. Dordrecht ; New York: Springer Verlag.

Mill, J., & Heijmans, B. T. (2013). From promises to practical strategies in epigenetic epidemiology. *Nature Reviews Genetics*, *14*(8), 585–594. https://doi.org/10.1038/nrg3405

Miller, E. M. (2009). Changes in serum immunity during pregnancy. *American Journal of Human Biology*, *21*(3), 401–403. https://doi.org/10.1002/ajhb.20882

Miller, E. M. (2010). Maternal hemoglobin depletion in a settled Northern Kenyan pastoral population. *American Journal of Human Biology*, *22*(6), 768–774. https://doi.org/10.1002/ajhb.21078

Miller, G. E., Yu, T., Chen, E., & Brody, G. H. (2015). Self-control forecasts better psychosocial outcomes but faster epigenetic aging in low-SES youth. *Proceedings of the National Academy of Sciences*, *112*(33), 10325–10330. https://doi.org/10.1073/pnas.1505063112

Min, K.-J., Lee, C.-K., & Park, H.-N. (2012). The lifespan of Korean eunuchs. *Current Biology*, *22*(18), R792–R793. https://doi.org/10.1016/j.cub.2012.06.036

Mitteldorf, J. (2014). Evolutionary Orthodoxy: How and Why the Evolutionary Theory of Aging Went Astray. *Current Aging Science*, *7*(1), 38–47. https://doi.org/10.2174/1874609807666140521105844

Moatt, J. P., Nakagawa, S., Lagisz, M., & Walling, C. A. (2016). The effect of dietary restriction on reproduction: A meta-analytic perspective. *BMC Evolutionary Biology*, *16*(1), 199. https://doi.org/10.1186/s12862-016-0768-z

Moore, D. S. (2015). *The developing genome: An introduction to behavioral epigenetics*. Oxford ; New York, NY: Oxford University Press.

Moore, S. R., Humphreys, K. L., Colich, N. L., Davis, E. G., Lin, D. T. S., MacIsaac, J. L., … Gotlib, I. H. (2020). Distinctions between sex and time in patterns of DNA methylation across puberty. *BMC Genomics*, *21*(1), 1–16. https://doi.org/10.1186/s12864-020-06789-3

Morange, M. (2006). Post-genomics, between reduction and emergence. *Synthese*, *151*(3), 355–360. https://doi.org/10.1007/s11229-006-9029-9

Morelli, S., Mandal, M., Goldsmith, L. T., Kashani, B. N., & Ponzio, N. M. (2015). The maternal immune system during pregnancy and its influence on fetal development. *Research and Reports in Biology*, 171. https://doi.org/10.2147/RRB.S80652

Morgan, T. H. (1910). Sex limited inheritance in drosophila. *Science*, *32*(812), 120. https://doi.org/10.1126/science.32.812.120

Morgan, Thomas Hunt. (1917). The theory of the gene. *American Naturalist*, 513–544.

Morris, T. J., & Beck, S. (2015). Analysis pipelines and packages for Infinium HumanMethylation450 BeadChip (450k) data. *Methods*, *72*, 3–8. https://doi.org/10.1016/j.ymeth.2014.08.011

Most, J., Tosti, V., Redman, L. M., & Fontana, L. (2017). Calorie restriction in humans: An update. *Ageing Research Reviews*, *39*, 36–45. https://doi.org/10.1016/j.arr.2016.08.005

Muehlenbein, Michael P., & Bribiescas, R. G. (2005). Testosterone-mediated immune functions and male life histories. *American Journal of Human Biology*, *17*(5), 527–558. https://doi.org/10.1002/ajhb.20419

Muehlenbein, Michael P., Hirschtick, J. L., Bonner, J. Z., & Swartz, A. M. (2010). Toward quantifying the usage costs of human immunity: Altered metabolic rates and hormone levels during acute immune activation in men. *American Journal of Human Biology*, *22*(4), 546–556. https://doi.org/10.1002/ajhb.21045

Muehlenbein, Micheal P. (2004). The immunosomatic metabolic diversion hypothesis and testosterone correlates with intestinal parasitemia in wild male chimpanzees. *American Journal of Physical Anthropology*, *123(S38)*, 150.

Muller, H. J. (1930). Oenothera-like linkage of chromosomes in Drosophila. *Journal of Genetics*, *22*(3), 335–357. https://doi.org/10.1007/BF02984196

Muller, M. N. (2017). Testosterone and reproductive effort in male primates. *Hormones and Behavior*, *91*, 36–51. https://doi.org/10.1016/j.yhbeh.2016.09.001

Mulligan, C. J. (2016). Early Environments, Stress, and the Epigenetics of Human Health. *Annual Review of Anthropology*, *45*(1), 233–249. https://doi.org/10.1146/annurev-anthro-102215-095954

Nanney, D. L. (1958). Epigenetic control systems. *Proceedings of the National Academy of Sciences*, *44*(7), 712–717.

Nelson, R. J. (2005). *An introduction to behavioral endocrinology*. Sinauer Associates.

Nelson, R. M., Pettersson, M. E., & Carlborg, Ö. (2013). A century after Fisher: Time for a new paradigm in quantitative genetics. *Trends in Genetics*, *29*(12), 669–676.

Nettle, D. (2010). Dying young and living fast: Variation in life history across English neighborhoods. *Behavioral Ecology*, *21*(2), 387–395. https://doi.org/10.1093/beheco/arp202

Ng, J. W., Barrett, L. M., Wong, A., Kuh, D., Smith, G. D., & Relton, C. L. (2012). The role of longitudinal cohort studies in epigenetic epidemiology: Challenges and opportunities. *Genome Biol*, *13*(6), 246.

Nieschlag, E., Behre, H. M., & Nieschlag, S. (Eds.). (2012). *Testosterone: Action, deficiency, substitution* (4ed.). Cambridge, UK: Cambridge University Press. Retrieved from http://gen.lib.rus.ec/book/index.php?md5=af8106cf43110ca7dc493a8ca1d0200c

Nieschlag, Eberhard, Nieschlag, S., & Behre, H. M. (1993). Lifespan and testosterone. *Nature*, *366*(6452), 215–215. https://doi.org/10.1038/366215a0

Non, A. L., Hollister, B. M., Humphreys, K. L., Childebayeva, A., Esteves, K., Zeanah, C. H., … Drury, S. S. (2016). Dna methylation at stress-related genes is

associated with exposure to early life institutionalization: DNA methylation and early adversity. *American Journal of Physical Anthropology*. https://doi.org/10.1002/ajpa.23010

Noordwijk, A. J. van, & Jong, G. de. (1986). Acquisition and Allocation of Resources: Their Influence on Variation in Life History Tactics. *The American Naturalist*, *128*(1), 137–142.

Norman, A. W., & Litwack, G. (1997). *Hormones* (2nd ed.). Cambridge, MA: Academic Press.

Nowak, J., Pawłowski, B., Borkowska, B., Augustyniak, D., & Drulis-Kawa, Z. (2018). No evidence for the immunocompetence handicap hypothesis in male humans. *Scientific Reports*, *8*(1), 7392. https://doi.org/10.1038/s41598-018-25694-0

Obeso, J. R. (2002). The costs of reproduction in plants. *New Phytologist*, *155*(3), 321–348. https://doi.org/10.1046/j.1469-8137.2002.00477.x

O'Callaghan, N., Bull, C., Palmer, L., Lyons, G., Graham, R., & Fenech, M. (2008). Buccal cells: A non-invasive measurement of selenium, zinc and magnesium status, and telomere length. *Asia Pac. J. Clin. Nutr*, *17*, S19.

O'Donovan, A., Pantell, M. S., Puterman, E., Dhabhar, F. S., Blackburn, E. H., Yaffe, K., … Body Composition, S. (2011). Cumulative Inflammatory Load Is Associated with Short Leukocyte Telomere Length in the Health, Aging and Body Composition Study. *PLoS ONE*, *6*(5), e19687.

Okada, Y., Teramura, K., & Takahashi, K. H. (2014). Heat shock proteins mediate trade-offs between early-life reproduction and late survival in Drosophila

melanogaster. *Physiological Entomology*, *39*(4), 304–312. https://doi.org/10.1111/phen.12076

Olovnikov, A. M. (1971). Principle of marginotomy in template synthesis of polynucleotides. *Dokl Akad Nauk SSSR*, *201*(6), 1496–1499.

Olsen, N. J., & Kovacs, W. J. (2002). Androgens in immunology and autoimmune diseases. In C. Chang (Ed.), *Androgens and androgen receptor: Mechanisms, functions, and clinical applications* (pp. 279–288). Norwell, MA: Springer.

Partridge, L., & Harvey, P. H. (1988). The Ecological Context of Life History Evolution. *Science*, *241*(4872), 1449–1455. https://doi.org/10.1126/science.241.4872.1449

Peña, E. D. L., Martín, J., Barja, I., Pérez-Caballero, R., Acosta, I., & Carranza, J. (2020). Immune challenge of mating effort: Steroid hormone profile, dark ventral patch and parasite burden in relation to intrasexual competition in male Iberian red deer. *Integrative Zoology*, *15*(4), 262–275. https://doi.org/10.1111/1749-4877.12427

Peng, C., Cardenas, A., Rifas-Shiman, S. L., Hivert, M.-F., Gold, D. R., Platts-Mills, T. A., … DeMeo, D. L. (2019). Epigenetic age acceleration is associated with allergy and asthma in children in Project Viva. *Journal of Allergy and Clinical Immunology*, *143*(6), 2263-2270.e14. https://doi.org/10.1016/j.jaci.2019.01.034

Penn, D. J., & Smith, K. R. (2007). Differential fitness costs of reproduction between the sexes. *Proceedings of the National Academy of Sciences*, *104*(2), 553–558. https://doi.org/10.1073/pnas.0609301103

Perez, T. L. (2015). *Attrition in the Cebu longitudinal health and nutrition survey* (No. Report Series No. 1). USC-Office of Population Studies Foundation, Inc. Retrieved from USC-Office of Population Studies Foundation, Inc website: http://www.opsusc.org/

Pidsley, R., Zotenko, E., Peters, T. J., Lawrence, M. G., Risbridger, G. P., Molloy, P., … Clark, S. J. (2016). Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. *Genome Biology*, *17*(1), 208. https://doi.org/10.1186/s13059-016-1066-1

Pillus, L., & Rine, J. (1989). Epigenetic inheritance of transcriptional states in S. cerevisiae. *Cell*, *59*(4), 637–647.

Plant, T. M., & Zeleznik, A. J. (2014). *Knobil and Neill's physiology of reproduction*. Academic Press.

Pollack, A. Z., Rivers, K., & Ahrens, K. A. (2018). Parity associated with telomere length among US reproductive age women. *Human Reproduction*, *33*(4), 736–744. https://doi.org/10.1093/humrep/dey024

Pommier, J.-P., Gauthier, L., Livartowski, J., Galanaud, P., Boué, F., Dulioust, A., … Boussin, F.-D. (1997). Immunosenescence in HIV Pathogenesis. *Virology*, *231*(1), 148–154.

Prall, S. P., & Muehlenbein, M. P. (2015). Dehydroepiandrosterone and multiple measures of functional immunity in young adults: DHEA and immune function in young adults. *American Journal of Human Biology*, *27*(6), 877–880. https://doi.org/10.1002/ajhb.22724

Prentice, A. M., & Prentice, A. (1988). Energy Costs of Lactation. *Annual Review of Nutrition*, *8*(1), 63–79. https://doi.org/10.1146/annurev.nu.08.070188.000431

Price, E. M., Cotton, A. M., Lam, L. L., Farré, P., Emberly, E., Brown, C. J., … Kobor, M. S. (2013). Additional annotation enhances potential for biologically-relevant analysis of the Illumina Infinium HumanMethylation450 BeadChip array. *Epigenetics & Chromatin*, *6*(1), 1.

Price, T., & Schluter, D. (1991). On the Low Heritability of Life-History Traits. *Evolution*, *45*(4), 853–861. https://doi.org/10.2307/2409693

Quach, A., Levine, M. E., Tanaka, T., Lu, A. T., Chen, B. H., Ferrucci, L., … others. (2017). Epigenetic clock analysis of diet, exercise, education, and lifestyle factors. *Aging (Albany NY)*, *9*(2), 419–446.

R Core Development Team. (2016). R: A language and environment for statistical computing, reference index version 3.3.1. *R Foundation for Statistical Computing, Vienna, Austria.*

Rakyan, V. K., Down, T. A., Balding, D. J., & Beck, S. (2011). Epigenome-wide association studies for common human diseases. *Nature Reviews Genetics*, *12*(8), 529–541.

Ramagopalan, S. V., & Rakyan, V. K. (2013). The promise and challenges of blood spot methylomics. *Epigenetics*, *8*(8), 775–777. https://doi.org/10.4161/epi.25357

Rando, O. J., & Verstrepen, K. J. (2007). Timescales of Genetic and Epigenetic Inheritance. *Cell*, *128*(4), 655–668. https://doi.org/10.1016/j.cell.2007.01.023

Rantala, M. J., Moore, F. R., Skrinda, I., Krama, T., Kivleniece, I., Kecko, S., & Krams, I. (2012). Evidence for the stress-linked immunocompetence handicap hypothesis in humans. *Nature Communications*, *3*, 694. https://doi.org/10.1038/ncomms1696

Rappaport, N., Twik, M., Plaschkes, I., Nudel, R., Iny Stein, T., Levitt, J., … Lancet, D. (2017). MalaCards: An amalgamated human disease compendium with diverse clinical and genetic annotation and structured search. *Nucleic Acids Research*, *45*(D1), D877–D887. https://doi.org/10.1093/nar/gkw1012

Réale, D., Garant, D., Humphries, M. M., Bergeron, P., Careau, V., & Montiglio, P.-O. (2010). Personality and the emergence of the pace-of-life syndrome concept at the population level. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1560), 4051–4063. https://doi.org/10.1098/rstb.2010.0208

Reimers, C. D., Knapp, G., & Reimers, A. K. (2012). Does Physical Activity Increase Life Expectancy? A Review of the Literature. *Journal of Aging Research*, *2012*. https://doi.org/10.1155/2012/243958

Rej, P. H., Bondy, M. H., Lin, J., Prather, A. A., Kohrt, B. A., Worthman, C. M., & Eisenberg, D. T. A. (2020). Telomere length analysis from minimally-invasively collected samples: Methods development and meta-analysis of the validity of different sampling techniques. *American Journal of Human Biology*, e23410. https://doi.org/10.1002/ajhb.23410

Reznick, D. (1985). Costs of reproduction: An evaluation of the empirical evidence. *Oikos*, *44*(2), 257–267.

Reznick, David, Nunney, L., & Tessier, A. (2000). Big houses, big cars, superfleas and the costs of reproduction. *Trends in Ecology & Evolution*, *15*(10), 421–425. https://doi.org/10.1016/S0169-5347(00)01941-8

Richter, T., & Zglinicki, T. (2007). A continuous correlation between oxidative stress and telomere shortening in fibroblasts. *Experimental Gerontology*, *42*(11), 1039–1042.

Ringbäck Weitoft, G., Burström, B., & Rosén, M. (2004). Premature mortality among lone fathers and childless men. *Social Science & Medicine*, *59*(7), 1449–1459. https://doi.org/10.1016/j.socscimed.2004.01.026

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–e47. https://doi.org/10.1093/nar/gkv007

Roberts, C. W., Satoskar, A., & Alexander, J. (1996). Sex steroids, pregnancy-associated hormones and immunity to parasitic infection. *Parasitology Today*, *12*(10), 382–388.

Robertson, S. A., Petroff, M. G., & Hunt, J. S. (2015). Chapter 41—Immunology of Pregnancy. In T. M. Plant & A. J. Zeleznik (Eds.), *Knobil and Neill's Physiology of Reproduction (Fourth Edition)* (pp. 1835–1874). San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-397175-3.00041-7

Rose, M. R. (1994). *Evolutionary biology of aging*. New York, NY: Oxford University Press.

Rose, M. R., Burke, M. K., Shahrestani, P., & Mueller, L. D. (2008). Evolution of ageing since Darwin. *Journal of Genetics*, *87*(4), 363–371. https://doi.org/10.1007/s12041-008-0059-6

Rose, M. R., Drapeau, M. D., Yazdi, P. G., Shah, K. H., Moise, D. B., Thakar, R. R., … Mueller, L. D. (2002). Evolution of late-life mortality in drosophila melanogaster. *Evolution*, *56*(10), 1982–1991.

Ryan, C. P. (2020). “Epigenetic clocks”: Theory and applications in human biology. *American Journal of Human Biology*, 18. https://doi.org/10.1002/ajhb.23488

Ryan, C. P., Brownlie, J. C., & Whyard, S. (2016). Hsp90 and physiological stress are linked to autonomous transposon mobility and heritable genetic change in nematodes. *Genome Biology and Evolution*, *8*(12), 3794–3805. https://doi.org/10.1093/gbe/evw284

Ryan, C. P., Hayes, M. G., Lee, N. R., McDade, T. W., Jones, M. J., Kobor, M. S., … Eisenberg, D. T. A. (2018). Reproduction predicts shorter telomeres and epigenetic age acceleration among young adult women. *Scientific Reports*, *8*(1), 11100. https://doi.org/10.1038/s41598-018-29486-4

Ryan, C. P., & Kuzawa, C. W. (2020). Germline epigenetic inheritance: Challenges and opportunities for linking human paternal experience with offspring biology and health. *Evolutionary Anthropology: Issues, News, and Reviews*, *29*(4), 180–200. https://doi.org/10.1002/evan.21828

Sampino, S., Stankiewicz, A. M., Zacchini, F., Goscik, J., Szostak, A., Swiergiel, A. H., … Ptak, G. E. (2017). Pregnancy at Advanced Maternal Age Affects Behavior and Hippocampal Gene Expression in Mouse Offspring. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *72*(11), 1465–1473. https://doi.org/10.1093/gerona/glx016

Sampson, M. J., Winterbone, M. S., Hughes, J. C., Dozio, N., & Hughes, D. A. (2006). Monocyte telomere shortening and oxidative DNA damage in type 2 diabetes. *Diabetes Care*, *29*(2), 283–289.

Sanders, J. L., Fitzpatrick, A. L., Boudreau, R. M., Arnold, A. M., Aviv, A., Kimura, M., … Newman, A. B. (2012). Leukocyte telomere length is associated with noninvasively measured age-related disease: The Cardiovascular Health Study. *J Gerontol A Biol Sci Med Sci*, *67*(4), 409–416.

Sandoval, J., Heyn, H., Moran, S., Serra-Musach, J., Pujana, M. A., Bibikova, M., & Esteller, M. (2011). Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics*, *6*(6), 692–702. https://doi.org/10.4161/epi.6.6.16196

Schmidt, E. E., Pelz, O., Buhlmann, S., Kerr, G., Horn, T., & Boutros, M. (2013). GenomeRNAi: A database for cell-based and in vivo RNAi phenotypes, 2013 update. *Nucleic Acids Research*, *41*(D1), D1021–D1026. https://doi.org/10.1093/nar/gks1170

Sear, R. (2020). Do human 'life history strategies' exist? *Evolution and Human Behavior*. https://doi.org/10.1016/j.evolhumbehav.2020.09.004

Seeman, T. E., McEwen, B. S., Rowe, J. W., & Singer, B. H. (2001). Allostatic load as a marker of cumulative biological risk: MacArthur studies of successful aging. *Proceedings of the National Academy of Sciences*, *98*(8), 4770–4775.

Shannon, P. (2003). Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Research*, *13*(11), 2498–2504. https://doi.org/10.1101/gr.1239303

Sharma, G., & Goodwin, J. (2006). Effect of aging on respiratory system physiology and immunology. *Clinical Interventions in Aging*, *1*(3), 253.

Shine, R. (1980). “Costs” of reproduction in reptiles. *Oecologia*, *46*(1), 92–100. https://doi.org/10.1007/BF00346972

Sidler, C., Kovalchuk, O., & Kovalchuk, I. (2017). Epigenetic Regulation of Cellular Senescence and Aging. *Frontiers in Genetics*, *8*. https://doi.org/10.3389/fgene.2017.00138

Sillanpää, E., Ollikainen, M., Kaprio, J., Wang, X., Leskinen, T., Kujala, U. M., & Törmäkangas, T. (2019). Leisure-time physical activity and DNA methylation age—A twin study. *Clinical Epigenetics*, *11*(1), 12. https://doi.org/10.1186/s13148-019-0613-5

Simanainen, U., Brogley, M., Gao, Y. R., Jimenez, M., Harwood, D. T., Handelsman, D. J., & Robins, D. M. (2011). Length of the human androgen receptor glutamine tract determines androgen sensitivity in vivo. *Molecular and Cellular Endocrinology*, *342*(1–2), 81–86. https://doi.org/10.1016/j.mce.2011.05.011

Simoni, M., Fanelli, F., Roli, L., & Pagotto, U. (2012). Measuring testosterone, DHT, and SHBG. In Eberhard Nieschlag, H. M. Behre, & S. Nieschlag (Eds.), *Testosterone: Action, deficiency, substitution* (4ed., pp. 60–86). Cambridge, UK: Cambridge University Press.

Simons, R. L., Lei, M. K., Beach, S. R. H., Philibert, R. A., Cutrona, C. E., Gibbons, F. X., & Barr, A. (2016). Economic hardship and biological weathering: The epigenetics of aging in a U.S. sample of black women. *Social Science & Medicine*, *150*, 192–200. https://doi.org/10.1016/j.socscimed.2015.12.001

Simpkin, A. J., Hemani, G., Suderman, M., Gaunt, T. R., Lyttleton, O., Mcardle, W. L., … Smith, G. D. (2016). Prenatal and early life influences on epigenetic age in children: A study of mother–offspring pairs from two cohort studies. *Human Molecular Genetics*, *25*(1), 191–201. https://doi.org/10.1093/hmg/ddv456

Simpkin, A. J., Howe, L. D., Tilling, K., Gaunt, T. R., Lyttleton, O., McArdle, W. L., … Relton, C. L. (2017). The epigenetic clock and physical development during childhood and adolescence: Longitudinal analysis from a UK birth cohort. *International Journal of Epidemiology*, *46*(2), 549–558. https://doi.org/10.1093/ije/dyw307

Singaraja, R. R., Tietjen, I., Hovingh, G. K., Franchini, P. L., Radomski, C., Wong, K., … Hayden, M. R. (2014). Identification of four novel genes contributing to familial elevated plasma HDL cholesterol in humans. *Journal of Lipid Research*, *55*(8), 1693–1701. https://doi.org/10.1194/jlr.M048710

Slieker, R. C., Relton, C. L., Gaunt, T. R., Slagboom, P. E., & Heijmans, B. T. (2018). Age-related DNA methylation changes are tissue-specific with ELOVL2 promoter methylation as exception. *Epigenetics & Chromatin*, *11*(1), 1–11. https://doi.org/10.1186/s13072-018-0191-3

Snyder-Mackler, N., Sanz, J., Kohn, J. N., Brinkworth, J. F., Morrow, S., Shaver, A. O., … Tung, J. (2016). Social status alters immune regulation and response to infection in macaques. *Science (New York, N.Y.)*, *354*(6315), 1041–1045. https://doi.org/10.1126/science.aah3580

Solomon, O., MacIsaac, J., Quach, H., Tindula, G., Kobor, M. S., Huen, K., … Holland, N. (2018). Comparison of DNA methylation measured by Illumina 450K and EPIC BeadChips in blood of newborns and 14-year-old children. *Epigenetics*, *13*(6), 655–664. https://doi.org/10.1080/15592294.2018.1497386

Solorio, S., Murillo-Ortíz, B., Hernández-González, M., Guillén-Contreras, J., Arenas-Aranda, D., Solorzano-Zepeda, F. J., … Malacara-Hernández, J. M. (2011). Association Between Telomere Length and C-Reactive Protein and the Development of Coronary Collateral Circulation in Patients with Coronary Artery Disease. *Angiology*, *62*(6), 467–472.

Soma-Pillay, P., Catherine, N.-P., Tolppanen, H., Mebazaa, A., Tolppanen, H., & Mebazaa, A. (2016). Physiological changes in pregnancy. *Cardiovascular Journal of Africa*, *27*(2), 89–94. https://doi.org/10.5830/CVJA-2016-021

Speakman, J., & Król, E. (2005). Limits to sustained energy intake IX: a review of hypotheses. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, *175*(6), 375–394.

Speakman, J. R., & Selman, C. (2003). Physical activity and resting metabolic rate. *The Proceedings of the Nutrition Society*, *62*(3), 621–634. https://doi.org/10.1079/PNS2003282

Spólnicka, M., Pośpiech, E., Adamczyk, J. G., Freire-Aradas, A., Pepłońska, B., Zbieć-Piekarska, R., … Branicki, W. (2018). Modified aging of elite athletes revealed by analysis of epigenetic age markers. *Aging (Albany NY)*, *10*(2), 241–252. https://doi.org/10.18632/aging.101385

Stearns, S. C. (1989). Trade-offs in life-history evolution. *Functional Ecology*, *3*(3), 259–268.

Stearns, Stephen C. (1992). *The Evolution of Life Histories*. Oxford University Press, USA.

Stearns, Stephen C., Byars, S. G., Govindaraju, D. R., & Ewbank, D. (2010). Measuring selection in contemporary human populations. *Nature Reviews Genetics*, *11*(9), 611–622. https://doi.org/10.1038/nrg2831

Stearns, Stephen C., & Koella, J. C. (1986). The Evolution of Phenotypic Plasticity in Life-History Traits: Predictions of Reaction Norms for Age and Size at Maturity. *Evolution*, *40*(5), 893–913. https://doi.org/10.2307/2408752

Stearns, Stephen C, & Koella, J. C. (2007). *Evolution in health and disease*. OUP Oxford.

Stelzer, G., Rosen, N., Plaschkes, I., Zimmerman, S., Twik, M., Fishilevich, S., … Lancet, D. (2016). The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses. *Current Protocols in Bioinformatics*, *54*(1), 1.30.1-1.30.33. https://doi.org/10.1002/cpbi.5

Stencel, A., & Crespi, B. (2013). What is a genome? *Molecular Ecology*, *22*(13), 3437–3443.

Stieglitz, J., Beheim, B. A., Trumble, B. C., Madimenos, F. C., Kaplan, H., & Gurven, M. (2015). Low mineral density of a weight-bearing bone among adult women in a high fertility population. *American Journal of Physical Anthropology*, *156*(4), 637–648. https://doi.org/10.1002/ajpa.22681

Stinson, S., Bogin, B., & O'Rourke, D. H. (Eds.). (2012). *Human Biology: An Evolutionary and Biocultural Perspective* (2 edition). Hoboken, NJ: Wiley-Blackwell.

Stubbs, T. M., Bonder, M. J., Stark, A.-K., Krueger, F., von Meyenn, F., Stegle, O., & Reik, W. (2017). Multi-tissue DNA methylation age predictor in mouse. *Genome Biology*, *18*, 68. https://doi.org/10.1186/s13059-017-1203-5

Sturtevant, A. H. (1913). The linear arrangement of six sex-linked factors in Drosophila, as shown by their mode of association. *Journal of Experimental Zoology*, *14*(1), 43–59. https://doi.org/10.1002/jez.1400140104

Suchard, M. (2015). Immunosenescence: Ageing of the immune system : review. *SA Pharmaceutical Journal*, *82*(8), 28–31.

Surani, M. A. H., Barton, S. C., & Norris, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*, *308*(5959), 548–550. https://doi.org/10.1038/308548a0

Sutton, W. S. (1902). On the morphology of the chromoso group in Brachystola magna. *The Biological Bulletin*, *4*(1), 24–39.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*, *131*(5), 861–872. https://doi.org/10.1016/j.cell.2007.11.019

Tamakoshi, A., Tamakoshi, K., Lin, Y., Mikami, H., Inaba, Y., Yagyu, K., & Kikuchi, S. (2011). Number of children and all-cause mortality risk: Results from the Japan Collaborative Cohort Study. *European Journal of Public Health*, *21*(6), 732–737. https://doi.org/10.1093/eurpub/ckq175

Teschendorff, A. E., Marabita, F., Lechner, M., Bartlett, T., Tegner, J., Gomez-Cabrero, D., & Beck, S. (2013). A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. *Bioinformatics*, *29*(2), 189–196. https://doi.org/10.1093/bioinformatics/bts680

Teschendorff, A. E., Zhuang, J., & Widschwendter, M. (2011). Independent surrogate variable analysis to deconvolve confounding factors in large-scale microarray profiling studies. *Bioinformatics*, *27*(11), 1496–1505.

Thomas, P. (2008). *Changes in buccal cytome biomarkers in relation to ageing and Alzheimer's disease.* (University of Adelaide). University of Adelaide. Retrieved from http://hdl.handle.net/2440/56186

Thompson, M. J., vonHoldt, B., Horvath, S., & Pellegrini, M. (2017). An epigenetic aging clock for dogs and wolves. *Aging (Albany NY)*, *9*(3), 1055–1068. https://doi.org/10.18632/aging.101211

Thurston, R. C., Carroll, J. E., Levine, M., Chang, Y., Crandall, C., Manson, J. E., … Horvath, S. (2020). Vasomotor Symptoms and Accelerated Epigenetic Aging in the Women's Health Initiative (WHI). *The Journal of Clinical Endocrinology & Metabolism*, *105*(4). https://doi.org/10.1210/clinem/dgaa081

Tikkanen, E., Gustafsson, S., Amar, D., Shcherbina, A., Waggott, D., Ashley, E. A., & Ingelsson, E. (2018). Biological Insights Into Muscular Strength: Genetic Findings in the UK Biobank. *Scientific Reports*, *8*(1), 1–9. https://doi.org/10.1038/s41598-018-24735-y

Tollefsbol, T. (Ed.). (2012). *Epigenetics in human disease* (1st ed.). San Diego, CA: Academic Press. Retrieved from https://books.google.com/books?hl=en&lr=&id=m18kGTHNSOkC&oi=fnd&pg=PP1&dq=%22of+epigenetic+diseases+because+their+reversibility+makes+these+diseases+amenable%22+%22disease+is+%EF%AC%81rst+described+in+terms+of+the+underlying+role+of+epigenetics+in+the%22+%22nature+of+the+disorders.+Chapters+are+also+presented+on+the+epigenetics+of%22+&ots=X--Cb_6hPI&sig=Wk5bVSGeYqn210qq3yTUOOWGEX4

Tollefsbol, T. O. (2012). Epigenetics of Human Disease. In *Epigenetics in Human Disease* (pp. 1–6). Elsevier. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/B9780123884152000019

Tracer, D. P. (1991). Fertility-related changes in maternal body composition among the au of Papua New Guinea. *American Journal of Physical Anthropology*, *85*(4), 393–405.

Trivers, R. L. (1972). Parental investment and sexual selection. In B. G. Campbell (Ed.), *Sexual Selection and the descent of man, 1871-1971*. Chicago, IL: Aldine Publishing Company. Retrieved from http://www.appstate.edu/~steelekm/classes/psy2664/Documents/trivers.pdf

Trumble, B. C., Blackwell, A. D., Stieglitz, J., Thompson, M. E., Suarez, I. M., Kaplan, H., & Gurven, M. (2016). Associations between male testosterone and immune function in a pathogenically stressed forager-horticultural population. *American Journal of Physical Anthropology*, n/a-n/a. https://doi.org/10.1002/ajpa.23054

Trumble, B. C., Jaeggi, A. V., & Gurven, M. (2015). Evolving the neuroendocrine physiology of human and primate cooperation and collective action. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1683), 20150014. https://doi.org/10.1098/rstb.2015.0014

Trundley, A., & Moffett, A. (2004). Human uterine leukocytes and pregnancy. *Tissue Antigens*, *63*(1), 1–12. https://doi.org/10.1111/j.1399-0039.2004.00170.x

Tung, J., Archie, E. A., Altmann, J., & Alberts, S. C. (2016). Cumulative early life adversity predicts longevity in wild baboons. *Nature Communications*, *7*, 11181. https://doi.org/10.1038/ncomms11181

Uhlén, M., Björling, E., Agaton, C., Szigyarto, C. A.-K., Amini, B., Andersen, E., … Pontén, F. (2005). A Human Protein Atlas for Normal and Cancer Tissues Based on Antibody Proteomics. *Molecular & Cellular Proteomics*, *4*(12), 1920–1932. https://doi.org/10.1074/mcp.M500279-MCP200

Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., … Pontén, F. (2015). Tissue-based map of the human proteome. *Science*, *347*(6220). https://doi.org/10.1126/science.1260419

Urlacher, S. S., Ellison, P. T., Sugiyama, L. S., Pontzer, H., Eick, G., Liebert, M. A., … Snodgrass, J. J. (2018). Tradeoffs between immune function and childhood growth among Amazonian forager-horticulturalists. *Proceedings of the National Academy of Sciences*, *115*(17), E3914–E3921. https://doi.org/10.1073/pnas.1717522115

Vaiserman, A. (2018). Developmental Tuning of Epigenetic Clock. *Frontiers in Genetics*, *9*. https://doi.org/10.3389/fgene.2018.00584

Vanderschueren, D., Sinnesael, M., Gielen, E., Claessens, F., & Boonen, S. (2012). Testosterone and bone. In Eberhard Nieschlag, H. M. Behre, & S. Nieschlag (Eds.), *Testosterone: Action, deficiency, substitution* (4ed., pp. 177–190). Cambridge, UK: Cambridge University Press.

Verhoeven, J. E., Yang, R., Wolkowitz, O. M., Bersani, F. S., Lindqvist, D., Mellon, S. H., … Hammamieh, R. (2018). Epigenetic Age in Male Combat-Exposed War Veterans: Associations with Posttraumatic Stress Disorder Status. *Molecular Neuropsychiatry*, *4*(2), 90–99. https://doi.org/10.1159/000491431

Verhulst, S., Susser, E., Factor-Litvak, P. R., Simons, M. J., Benetos, A., Steenstrup, T., … Aviv, A. (2015). Commentary: The reliability of telomere length measurements. *Int J Epidemiol*, *44*(5), 1683–1686.

Vidal-Bralo, L., Lopez-Golan, Y., & Gonzalez, A. (2016). Simplified Assay for Epigenetic Age Estimation in Whole Blood of Adults. *Frontiers in Genetics*, *7*. https://doi.org/10.3389/fgene.2016.00126

Villota-Salazar, N. A., Mendoza-Mendoza, A., & González-Prieto, J. M. (2016). Epigenetics: From the past to the present. *Frontiers in Life Science*, *9*(4), 347–370. https://doi.org/10.1080/21553769.2016.1249033

Waddington, C. H. (1942). The epigenotype. *Endeavour*, *1*, 18–20.

Walker, R. M., MacGillivray, L., McCafferty, S., Wrobel, N., Murphy, L., Kerr, S. M., … Evans, K. L. (2019). Assessment of dried blood spots for DNA methylation profiling. *Wellcome Open Research*, *4*, 44. https://doi.org/10.12688/wellcomeopenres.15136.1

Wallace, A. R. (1871). *Contributions to the theory of natural selection*. Macmillan. Retrieved from http://books.google.com/books?hl=en&lr=&id=qDwDAAAAQAAJ&oi=fnd&pg=PR1&dq=%22shown+that+the+present+state+of+the+earth+and+of+the%22+%22t

o+those+changes+(as+has+frequently+been+done)+must+lead%22+%22perhaps+hundreds,+perhaps+thousands+of+times:+%E2%80%94+That+all%22+&ots=jRBn5SSNiY&sig=-xgdSimqhqYEOW6mo8S1ogRz1-Q

Wang, Z., Wu, X., & Wang, Y. (2018). A framework for analyzing DNA methylation data from Illumina Infinium HumanMethylation450 BeadChip. *BMC Bioinformatics*, *19*(S5). https://doi.org/10.1186/s12859-018-2096-3

Watson, J. D., & Crick, F. H. (1953). A structure for deoxyribose nucleic acid. *Nature*, *171*(4356), 737–738.

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., … Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature Neuroscience*, *7*(8), 847–854. https://doi.org/10.1038/nn1276

Weidner, C. I., Lin, Q., Koch, C. M., Eisele, L., Beier, F., Ziegler, P., … Wagner, W. (2014). Aging of blood can be tracked by DNA methylation changes at just three CpG sites. *Genome Biology*, *15*, R24. https://doi.org/10.1186/gb-2014-15-2-r24

Weismann, A. (1891). *Essays upon heredity and kindred biological problems* (Vol. 1; E. B. Poulton & A. E. Shipley, Eds.). Clarendon press.

Weismann, A. (1893). *The germ-plasm: A theory of heredity*. New York, NY: C. Scribner's sons.

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. Oxford University Press.

Westendorp, R. G. J., & Kirkwood, T. B. L. (1998). Human longevity at the cost of reproductive success. *Nature*, *396*(6713), 743–746. https://doi.org/10.1038/25519

White House Press Release. (2000). White House Briefing. *Genome.Gov*. Retrieved from https://www.genome.gov/10001356/june-2000-white-house-event

Wickham, H., & Chang, W. (2013). *Package 'ggplot2.'*

Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C.-P., Nicholl, J. K., … Goodwin, R. G. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*, *3*(6), 673–682. https://doi.org/10.1016/1074-7613(95)90057-8

Wilkins, M., Stokes, A., & Wilson, H. (2003). Molecular structure of deoxypentose nucleic acids. *Nature*, *421*(6921), 398–400.

Williams, G. C. (1957). Pleiotropy, Natural Selection, and the Evolution of Senescence. *Evolution*, 398–411.

Williams, G. C. (1966a). *Adaptation and natural selection: A critique of some current evolutionary thought*. Princeton, NJ: Princeton Univ. Press.

Williams, G. C. (1966b). Natural Selection, the Costs of Reproduction, and a Refinement of Lack's Principle. *The American Naturalist*, *100*(916), 687–690.

Wilson, E. O. (2000). *Sociobiology: The new synthesis*. Harvard University Press.

Wilson, M., & Daly, M. (1985). Competitiveness, risk taking, and violence: The young male syndrome. *Ethology and Sociobiology*, *6*(1), 59–73.

Winkler, T. W., Justice, A. E., Graff, M., Barata, L., Feitosa, M. F., Chu, S., … Loos, R. J. F. (2015). The Influence of Age and Sex on Genetic Associations with Adult Body Size and Shape: A Large-Scale Genome-Wide Interaction Study. *PLOS Genetics*, *11*(10), e1005378. https://doi.org/10.1371/journal.pgen.1005378

Witkowska-Zimny, M., & Kaminska-El-Hassan, E. (2017). Cells of human breast milk. *Cellular & Molecular Biology Letters*, *22*(1), 11. https://doi.org/10.1186/s11658-017-0042-4

Woltereck, R. (1909). Weitere experimentelle Unter-suchungen uber Artveranderung, speziell uber das Wesen quantitativer Artunterschiede bei Daphniden. *Verh. D. Tsch. Zool. Ges.*, *1909*, 110–172.

World Health Organization. (2015). *World report on ageing and health*. World Health Organization.

Wu, Y., McDade, T. W., Kuzawa, C. W., Borja, J., Li, Y., Adair, L. S., … Lange, L. A. (2012). Genome-wide Association with C-Reactive Protein Levels in CLHNS: Evidence for the CRP and HNF1A Loci and their Interaction with Exposure to a Pathogenic Environment. *Inflammation*, *35*(2), 574–583. https://doi.org/10.1007/s10753-011-9348-y

Yang, J., Zaitlen, N. A., Goddard, M. E., Visscher, P. M., & Price, A. L. (2014). Advantages and pitfalls in the application of mixed-model association methods. *Nature Genetics*, *46*(2), 100–106. https://doi.org/10.1038/ng.2876

Yeap, B. B., Knuiman, M. W., Divitini, M. L., Hui, J., Arscott, G. M., Handelsman, D. J., … Beilby, J. P. (2016). Epidemiological and Mendelian randomisation studies of

dihydrotestosterone and estradiol, and leucocyte telomere length in men. *The Journal of Clinical Endocrinology & Metabolism*, jc.2015-4139.

Zahavi, A. (1975). Mate selection—A selection for a handicap. *Journal of Theoretical Biology*, *53*(1), 205–214.

Zannas, A. S., Arloth, J., Carrillo-Roa, T., Iurato, S., Röh, S., Ressler, K. J., … 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

Zarulli, V., Jones, J. A. B., Oksuzyan, A., Lindahl-Jacobsen, R., Christensen, K., & Vaupel, J. W. (2018). Women live longer than men even during severe famines and epidemics. *Proceedings of the National Academy of Sciences*, 201701535. https://doi.org/10.1073/pnas.1701535115

Zeilinger, S., Kühnel, B., Klopp, N., Baurecht, H., Kleinschmidt, A., Gieger, C., … Illig, T. (2013). Tobacco Smoking Leads to Extensive Genome-Wide Changes in DNA Methylation. *PLOS ONE*, *8*(5), e63812. https://doi.org/10.1371/journal.pone.0063812

Zeng, J., Konopka, G., Hunt, B. G., Preuss, T. M., Geschwind, D., & Yi, S. V. (2012). Divergent Whole-Genome Methylation Maps of Human and Chimpanzee Brains Reveal Epigenetic Basis of Human Regulatory Evolution. *The American Journal of Human Genetics*, *91*(3), 455–465. https://doi.org/10.1016/j.ajhg.2012.07.024

Zeng, Y., Ni, Z., Liu, S., Gu, X., Huang, Q., Liu, J., & Wang, Q. (2016). Parity and All-cause Mortality in Women and Men: A Dose-Response Meta-Analysis of Cohort Studies. *Scientific Reports*, *6*(1), 19351. https://doi.org/10.1038/srep19351

Zhang, Q., Vallerga, C. L., Walker, R. M., Lin, T., Henders, A. K., Montgomery, G. W., … Visscher, P. M. (2019). Improved precision of epigenetic clock estimates across tissues and its implication for biological ageing. *Genome Medicine*, *11*(1), 54. https://doi.org/10.1186/s13073-019-0667-1

Zhang, Y., Saum, K.-U., Schöttker, B., Holleczek, B., & Brenner, H. (2018). Methylomic survival predictors, frailty, and mortality. *Aging*. https://doi.org/10.18632/aging.101392

Zhang, Y., Wilson, R., Heiss, J., Breitling, L. P., Saum, K.-U., Schöttker, B., … Brenner, H. (2017). DNA methylation signatures in peripheral blood strongly predict all-cause mortality. *Nature Communications*, *8*, 14617. https://doi.org/10.1038/ncomms14617

Zhao, W., Ammous, F., Scott Ratliff, Liu, J., Yu, M., Mosley, T. H., … Smith, J. A. (2019). Education and Lifestyle Factors Are Associated with DNA Methylation Clocks in Older African Americans. *International Journal of Environmental Research and Public Health*, *16*(17), 3141. https://doi.org/10.3390/ijerph16173141

Zheng, Y., Joyce, B. T., Colicino, E., Liu, L., Zhang, W., Dai, Q., … Hou, L. (2016). Blood Epigenetic Age may Predict Cancer Incidence and Mortality. *EBioMedicine*, *5*, 68–73. https://doi.org/10.1016/j.ebiom.2016.02.008

Zhou, Q., Li, M., Wang, X., Li, Q., Wang, T., Zhu, Q., … Li, X. (2012). Immune-related MicroRNAs are Abundant in Breast Milk Exosomes. *International Journal of Biological Sciences*, *8*(1), 118–123. https://doi.org/10.7150/ijbs.8.118

Ziomkiewicz, A., Frumkin, A., Zhang, Y., Sancilio, A., & Bribiescas, R. G. (2018). The cost of reproduction in women: Reproductive effort and oxidative stress in premenopausal and postmenopausal American women. *American Journal of Human Biology*, *30*(1), n/a-n/a. https://doi.org/10.1002/ajhb.23069

Ziomkiewicz, A., Sancilio, A., Galbarczyk, A., Klimek, M., Jasienska, G., & Bribiescas, R. G. (2016). Evidence for the cost of reproduction in humans: High lifetime reproductive effort is associated with greater oxidative stress in post-menopausal women. *PloS One*, *11*(1), e0145753.

Ziomkiewicz, A., Wichary, S., & Jasienska, G. (2019). Cognitive costs of reproduction: Life-history trade-offs explain cognitive decline during pregnancy in women. *Biological Reviews*, *94*(3), 1105–1115. https://doi.org/10.1111/brv.12494

Zuk, O., Hechter, E., Sunyaev, S. R., & Lander, E. S. (2012). The mystery of missing heritability: Genetic interactions create phantom heritability. *Proceedings of the National Academy of Sciences*, *109*(4), 1193–1198. https://doi.org/10.1073/pnas.1119675109