DEVELOPMENTAL PLASTICITY: EARLY LIFE ENVIRONMENT, DNA METHYLATION, AND LATER LIFE PHENOTYPE IN SPOTTED HYENAS.

By

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ABSTRACT

DEVELOPMENTAL PLASTICITY: EARLY LIFE ENVIRONMENT, DNA METHYLATION, AND LATER LIFE PHENOTYPE IN SPOTTED HYENAS.

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Animals, including humans, vary in their phenotypes. While this phenomenon is partially explained by genotype, an organism's environment (and its experiences within a given environment) also contributes to trait variability. Of particular relevance to my dissertation is the notion of *developmental plasticity*, or variability in an organism's phenotype due to specific environmental exposures experienced across ontogeny. In my dissertation, I discuss theory (Chapter 2) and test empirical hypotheses (Chapters 3-5) regarding the role of developmental plasticity via DNA methylation in the relationship between early life environment and adult phenotype. Leveraging a wild population of spotted hyenas in the Masai Mara, Kenya (The Mara Hyena Project), my analytical Chapters 3-5 specifically focus on social and ecological measures of the early life environment in relation to global (via the luminometric assay [LUMA] of %CCGG methylation) and genome-wide DNA methylation (via Enhanced Reduced Representation Bisulfite Sequencing [ERRBS]). I then explore associations of each with adult stress phenotype, as indicated by fecal corticosterone concentrations. In Chapter 3, I detected differences in %CCGG methylation across the hyena genome with respect to both social and ecological factors early in life, suggesting that these characteristics may impact future phenotype through epigenetic modifications like global DNA methylation. In Chapter 4, I found that higher maternal care and greater social connectedness during early life are associated with higher %CCGG methylation and lower fecal corticosterone concentrations during adulthood, both of which are indicators of positive health. Finally, in Chapter 5, I identified differential methylation in CpG sites involved in inflammatory processes that were associated with both early life maternal care and adult fecal corticosterone levels, thereby implicating these sites as

possible mediators to the relationship between early life maternal care quality/quantity and future stress phenotype.

These findings are an important extension to current understanding of developmental plasticity that stems from rodent models and studies of captive primates. Biomedical models of developmental plasticity often utilize laboratory rodents, which lack the natural social structure and intact life history involved in development of complex and variable phenotypes. Studies of captive primates typically focus on extreme measures of early life social deprivation that do not capture the naturally occurring spectrum of social experiences in wild mammals. In wild spotted hyenas, I was able to test hypotheses surrounding the concept of developmental plasticity in a gregarious mammal with a complex social structure that is also subject to natural selection. Furthermore, use of data and samples from The Mara Hyena Project enabled me to characterize multiple aspects of the early life environment, DNA methylation, and stress phenotypes in the same individuals - a feat rarely accomplished even in well-established human cohort studies.

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CHAPTER 1

INTRODUCTION AND OVERVIEW OF THE DISSERTATION

INTRODUCTION

General Introduction

Animals, including humans, vary in their phenotypes. Throughout time, this variation has piqued the interest of scientists and inspired the creations of artists. Evolutionary biologists have looked for proximate and ultimate explanations for traits observed in the animals they study, and biomedical researchers have angled to understand and improve human health. At the root of their inquiry is the attempt to partition phenotypic variation into its constituent parts. In its simplest form, phenotypic variation (V_P) in a population can be described as the sum of variation in genotypes (V_G) plus the sum of variation in environments (V_E) (Conner & Hartl, 2004).

Following the modern synthesis, which married Mendelian genetics with Darwinian evolution, ensued a rapid expansion in our understanding of how genetic variation contributes to phenotypic variation; specifically, the DNA with which an organism is born was recognized as the raw substrate in the process of evolution by natural selection. Nevertheless, only part of observed phenotypic variation is attributable to genetic differences (Eichler et al., 2010), suggesting a role of the environment in shaping phenotypes. Thus, although genes and the non-coding regions of genomes are the raw materials upon which phenotypes are built, and therefore, a critical source of phenotypic variation, this fundamental property of organisms does not exclude the fact that the environment in which a genotype operates also matters. Where, when, and to what extent genes are expressed are properties that are influenced by the

environment, thereby rendering the environment an indelible contributor to phenotypic variation (G. E. Robinson, 2004).

Consider an analogy involving a collection of painted artworks. Each painting is unique and the variation in paintings is similar to the phenotypic variation we observe in a population of animals. The raw materials needed to create a painting include a canvas as well as some collection of colored paints, which corresponds to the collection of protein coding genes in a genome. Also included in this assortment of raw materials are paint brushes, which transport the various combinations of paint to the canvas, and basic instructions, such as the use of yellow and blue paints in combination to create green on the canvas. Paint brushes and simple instructions correspond to the non-coding regulatory regions of the genome, which similarly help transport and direct production of proteins from its DNA source. Variation in canvas, paint colors, and paint brushes will certainly affect the final painting. However, art is much more than these raw materials. Like the environment, which provides the context in which genes function, contextual factors that influence the artist will undoubtedly shape the artwork. Variation among the artists holding the paint brush, as well as factors that influence their mood and their decisions about where, when and how much paint to apply to the canvas, such as current lighting conditions or their social relationships, will each contribute to the final form of the painting. In both painted works of art and the phenotypes of organisms, the environment plays an important role.

Variation in phenotypic traits in animals that is attributable to the environment, and not solely their genotype, is known as phenotypic plasticity, a concept formalized by Mary West-Eberhard in the late 1980s (West-Eberhard, 1989). A specific case of phenotypic plasticity,

known as developmental plasticity, focuses on the ways in which early life exposure to environmental factors induces phenotypic variation later in life (Stamps, 2016). A parallel to developmental plasticity that exists in the biomedical realm is known as the Developmental Origins of Health and Disease (DOHaD) (Gillman, 2005). The DOHaD hypothesis emerged following a landmark study, led by epidemiologist David Barker, who observed that individuals who were underweight as infants had higher mortality risk from heart disease as adults (Barker, Osmond, Winter, Margetts, & Simmonds, 1989). To date, the concepts of DOHaD and developmental plasticity are prominent areas of research among evolutionary biologists and biomedical scientists alike (Bateson et al., 2004; Gilbert & Epel, 2015; Lea, Tung, Archie, & Alberts, 2017), as both groups strive to better understand the complex ways in which the early life environment imparts persistent effects on later life phenotypes.

Dissertation overview

In this dissertation, I explore theory and empirically test hypotheses related to developmental plasticity. Collectively, my thesis is concerned with elucidating ways in which the early life environment, molecular biomarkers such as DNA methylation, and later life phenotypes interact during the course of development. I focus on social and ecological measures of the early life environment in relation to DNA methylation as the molecular biomarker of interest, and the adult stress phenotype as the outcome of interest. Both social experience (Szyf, McGowan, & Meaney, 2008) and nutrition (Laubach, Faulk, Cardenas, & Perng, 2017) are important components of the early life environment known to be associated with patterns of DNA methylation. Briefly, mammalian DNA methylation refers to the

mitotically heritable, covalently bonded methyl groups on the fifth carbon of cytosine-guanine (CpG) dinucleotide pairs (Razin & Riggs, 1980), which are responsive to environmental stimuli and are also associated with gene expression (Klose & Bird, 2006; E. Li & Bird, 2007). Finally, I direct my attention to the stress phenotype because early life social environments (Adkins-Regan, 2005; McEwen & Seeman, 1999; Sapolsky, 2004) and DNA methylation (Hunter & McEwen, 2013) are associated with physiological and behavioral stress responses in adulthood in laboratory animals.

Overview of the study population

The empirical data within this dissertation come from a long-term field study of wild spotted hyenas (*Crocuta crocuta*) inhabiting the Masai Mara National Reserve, Kenya. Continuous behavioral, demographic, and ecological data have been collected since 1988 from individually identifiable hyenas. The study population comprises approximately six unique social groups, or clans, of hyenas. Spotted hyenas offer a good model for studying how early life social environments are associated with later life phenotypes because they live in large groups, reaching over 120 individuals (Green, Johnson-Ulrich, Couraud, & Holekamp, 2018), and they exhibit a wide range of social behaviors. These gregarious animals live in a complex social milieu, as clans are organized based on a linear, stable social hierarchy (Kruuk, 1972) in which an individual's social rank determines its priority of access to food and other resources that affect its fitness (Strauss & Holekamp, 2019; Tilson & Hamilton, 1984). Social dominance rank is passed on from mother to offspring in a cercopithecine primate-like pattern of maternal rank 'inheritance' with youngest rank ascendancy, even though there is no literal inheritance of rank

(Engh, Esch, Smale, & Holekamp, 2000). Finally, female hyenas invest heavily in their dependent young throughout the offspring's protracted period of development; this investment ranges from time and energy spent nursing to facilitating access to solid food until their young are approximately three years of age (Holekamp & Smale, 1998; Watts, Tanner, Lundrigan, & Holekamp, 2009).

Analytical approach overview

The analytical approaches that I employ in this dissertation borrow heavily from the principals of modern epidemiology (Rothman, Greenland, & Lash, 2008). Given that the data used here are largely observational, and that I am interested in understanding the etiology of adult phenotypes, I approach my analyses following A.B. Hill's (1965) causal criteria, and by structuring my research questions within the DOHaD framework using life-course epidemiological methods (Ben-shlomo & Kuh, 2002; Gage, Munafò, & Davey Smith, 2016; Gluckman, Hanson, & Buklijas, 2010; Kuh, Ben-Shlomo, Lynch, Hallqvist, & Power, 2003). Throughout my analytical chapters, I use Directed Acyclic Graphs (DAGs) to 1) explicitly define study questions, 2) to map out the causal relationships between independent and dependent variables, and 3) to summarize the analytical strategy. In general, DAGs include a series of nodes or variables that are connected by unidirectional arrows or edges in such a way as to convey the hypothesized causal relationship between variables, i.e. the effect X on Y (Greenland & Pearl, 2017; Greenland, Pearl, & Robins, 1999). DAGs are also useful for identifying variables that might bias the association(s) of interest, known as confounding variables (Robins, 2016; Shrier & Platt, 2008); variables that change the nature of the

association of interest, known as effect modifiers; and variables that are on the causal pathway between the X and Y variables, known as mediators. Given that each of these variables are treated differently in the analysis, explicitly mapping out the hypothesized causal relationships using a DAG serves as a point of reference for the biological narrative and provides a template for the analytical strategy.

DISSERTATION SUMMARY

Outline of dissertation chapters

There are five chapters in this dissertation including this introductory first chapter. **Chapter 2** is titled '*Epigenetics and the maintenance of developmental plasticity: extending the signalling theory framework*.' In this work, we adopt a signaling theory approach to explore developmental plasticity as a process that is both adaptive and subject to error. In this chapter we introduce the basic biology of DNA methylation and consider this molecular biomarker as a potential mechanism that facilitates developmental plasticity. This chapter was published in *Biological Reviews* in 2018. **Chapter 3** is titled '*Early life social and ecological determinants of global DNA methylation in wild spotted hyenas*.' In this chapter, we investigate the extent to which early life social and ecological variables are associated with patterns of global (%CCGG) DNA methylation in a large sample of spotted hyenas. Taking advantage of archived blood samples collected in cub, subadult and adult hyenas, we also inquire whether associations between global DNA methylation and socioecological variables are evident during different developmental stages. This chapter was published in *Molecular Ecology* in 2019. **Chapter 4** is titled '*Associations of maternal care and early life social experience with offspring DNA*

methylation and later life stress phenotype.' Here, we are interested in determining the extent to which maternal care in the first year of life, as well as social connections during two key early life developmental stages, are associated with adult stress phenotypes, and the possibility that this relationship is mediated by DNA methylation. **Chapter 5** is titled '*An exploratory analysis of functional genomic regions involved in the early life social environment and adult stress phenotype: A genome-wide association study using Enhanced Reduced Representation Bisulfite Sequencing (ERRBS) data*.' Here we aim to identify functional gene regulatory pathways through which early life social factors might influence later life stress phenotypes. We conduct an epigenome-wide association study (EWAS) to identify differentially methylated CpG sites that are associated with later life stress phenotypes in subadult females. We further home in on CpG sites of interest by identifying differentially methylated sites with respect to maternal social rank and receipt of maternal care during the year in which offspring were born. I further summarize Chapters 2-5 below.

Chapter summaries

Chapter 2: *Epigenetics and the maintenance of developmental plasticity: extending the signalling theory framework.*

Developmental plasticity, a phenomenon of importance in both evolutionary biology and human studies of the developmental origins of health and disease (DOHaD), enables organisms to respond to their environment based on early-life experience without changes to the underlying nucleotide sequence. Although such phenotypic responses should theoretically improve an organism's fitness and performance in its future environment, this is not always the

case. We first discuss epigenetics as an adaptive mechanism of developmental plasticity and use signaling theory to provide an evolutionary context for DOHaD phenomena within a generation. Next, we utilize signaling theory to identify determinants of adaptive developmental plasticity, detect sources of random variability – also known as process errors that affect maintenance of an epigenetic signal (DNA methylation) over time, and discuss implications of these errors for an organism's health and fitness. Finally, we apply life course epidemiology conceptual models to inform study design, and analytical strategies that are capable of parsing out the potential effects of process errors in relationships among an organism's early environment, DNA methylation, and phenotype in a future environment. Ultimately, we hope to foster cross talk and interdisciplinary collaboration between evolutionary biology and DOHaD epidemiology, which have historically remained separate despite a shared interest in developmental plasticity.

Chapter 3: *Early life social and ecological determinants of global DNA methylation in wild spotted hyenas*.

Environmental factors early in life can have lasting influence on the development and later-life phenotypes of animals, but the underlying molecular mechanisms mediating these phenomena remain poorly understood. We examined cross-sectional associations between early life socioecological factors and global (CCGG) DNA methylation in 293 wild spotted hyenas in Kenya, grouped into one of three age classes (cub, subadult, and adult). Explanatory variables of interest included maternal rank based on outcomes of dyadic agonistic interactions, litter size, wild ungulate prey density, and anthropogenic disturbance during the year in which each

hyena was born based on counts of livestock grazing illegally in the Reserve. The dependent variable of interest was global DNA methylation, assessed via the LUminometric Methylation Assay, which provides a % methylation value calculated at CCGG sites across the genome. Among cubs, we observed approximately 2.75% higher CCGG methylation in offspring born to high than low ranking mothers. Among cubs and subadults, higher anthropogenic disturbance corresponded with greater %CCGG methylation. In both cubs and adults, we found an inverse association between prey density measured before a hyena was three months old and %CCGG methylation. Our results suggest that maternal rank, anthropogenic disturbance, and prey availability early in life are associated with later life global DNA methylation. Future studies are required to understand the extent to which these DNA methylation patterns relate to adult phenotypes and fitness outcomes.

Chapter 4: *Associations of maternal care and early life social experience with offspring DNA methylation and later life stress phenotype.*

It is well-stablished that the early life social environment influences later life stress phenotypes, yet the underlying biological pathways are poorly understood. Using Baron & Kenny's method for mediation analysis, we tested the hypothesis that quality of maternal care and early life social connections with group mates are associated with adult baseline glucocorticoid concentrations, and that this relationship operates through global and targeted gene (Glucocorticoid Receptor) DNA methylation in a population of wild hyenas. When we examined associations between the early social environment (as indicated by maternal care behaviors and maternal rank) and adult stress phenotype (as indicated by fecal corticosterone

levels), we found that receipt of more grooming from one's mother during the first year of life, and higher social network connectivity (degree) during the den independent stage of development, were each associated with a more favorable adult stress phenotype, as indicated by lower fecal corticosterone levels. Next, an analysis of the relationship between the early life social environment and global DNA methylation revealed that spending more time in close proximity with one's mother, and more time spent nursing were related to higher global DNA methylation during the subadult and adult life stages. We also found that higher measures of social network degree and strength during the den independent, but not communal den, stage of development were associated with higher global DNA methylation. Having confirmed that the early social environment was associated with global DNA methylation, we next inquired whether global DNA methylation was related to adult stress phenotype. However, our analysis revealed no evidence of this, suggesting that global DNA methylation *per se* does not directly mediate the observed relationship between early social environment and adult stress phenotype in this study sample. We found near zero percent mean and overall low variation in CpG sites in the putative glucocorticoid promoter region of hyenas. These findings inspired the inquiries in Chapter 5, which investigates genome-wide DNA methylation patterns to identify specific gene regulatory pathways that might link early experience to later life stress.

Chapter 5: *An exploratory analysis of functional genomic regions involved in the early life social environment and adult stress phenotype: A genome-wide association study using Enhanced Reduced Representation Bisulfite Sequencing (ERRBS) data.*

In this final chapter, we conducted an epigenome-wide association study (EWAS) to home in on functional regions of the hyena genome that were first associated with later life stress phenotype and secondly were associated with maternal social rank and maternal care during the first year of life. In this exploratory analysis, our sample included 30 subadult female hyenas, from which we extracted genomic DNA from whole blood to generate ERRBS data. From these data we generated estimates of DNA methylation at a single nucleotide resolution at approximately 2.2 million CpG sites across the hyena genome. After false discovery rate correction, the EWAS revealed 15 CpG sites that were differentially methylated with respect to fecal corticosterone levels. Among these 15 sites, there were three CpG sites in which DNA methylation was associated with maternal care, with beta estimates in the anticipated direction given that we expected better maternal care to be associated with lower fecal corticosterone. These three CpG sites mapped near to genes in the human genome that have been implicated in stress-related inflammation pathways. This pioneering analysis paves the way for future studies to formally assess these CpG sites as mediating biomarkers between early social experience and later life stress phenotypes.

CHAPTER 2

EPIGENETICS AND THE MAINTENANCE OF DEVELOPMENTAL PLASTICITY: EXTENDING THE SIGNALLING THEORY FRAMEWORK

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INTRODUCTION

An individual's genotype is established at conception. Nevertheless, diverse phenotypes may arise from a single genotype in response to an organism's environment. This phenomenon, broadly referred to as 'phenotypic plasticity' (West-Eberhard, 1989), can occur in response to previous environmental exposures, known as 'developmental plasticity', or to concurrent exposures, known as 'contextual plasticity' (Stamps, 2016). Herein, we focus on developmental plasticity, both as an adaptive response (e.g. modified behaviour, physiology, or structure) that improves an organism's fitness in its projected future environment, and as a non-adaptive consequence of environmental instability and perturbed developmental processes that lead to a mismatch between an organism's phenotype and future environment (Stearns, 1989; West-Eberhard, 1989; Bateson *et al.*, 2004; Bateson, Gluckman & Hanson, 2014; Ghalambor *et al.*, 2007; Nettle & Bateson, 2015). Using signal system and life course epidemiology frameworks, we explore the temporal relationships among key components of developmental plasticity – namely, the environment experienced by an organism during early life, epigenetics as a mediating biological mechanism, and the organism's phenotype in the future environment. In

addition, we consider the role of stochasticity in endogenous plasticity, which is phenotypic variation due to the organism's changing internal state as it ages (Pigliucci, 1998; Stamps, 2016), as a potential source of error in epigenetic processes that underlie developmental plasticity.

Our objectives are threefold. First, we provide a brief summary of the relevant empirical evidence for epigenetic mediation of developmental plasticity in evolutionary developmental biology and in human studies of developmental origins of health and disease (DOHaD). Second, we use a signalling theory framework to illustrate how one specific epigenetic mechanism, DNA methylation, facilitates developmental plasticity. The bulk of this objective focuses on aligning epigenetic mechanisms (proximate explanations) of developmental plasticity with health- and fitness-related outcomes (ultimate explanations) in order to provide an evolutionary context for DOHaD phenomena. Finally, we explore the role of process error in epigenetic maintenance of developmental plasticity in both evolutionary developmental biology and human health using concepts from signalling theory and life course epidemiology framework.

EMPIRICAL EVIDENCE OF EPIGENETIC SIGNALLING

Epigenetic mechanisms, such as DNA methylation, are relatively stable, mitotically heritable changes in chromosomes that influence the phenotype of an organism within a generation, but are not due to alterations in the DNA nucleotide sequence (Allis, Jenuwein, & Reinberg, 2007; Berger, Kouzarides, Shiekhattar, & Shilatifard, 2009). Our focus is on DOHaD over the life course within a generation, rather than on meiotically stable maternal effects across generations (see McNamara *et al.*, 2016 and references therein). Of particular relevance to this review is the

fact that epigenetic changes to the DNA may facilitate developmental plasticity by encoding information from an organism's early life, including the prenatal environment, to coordinate future gene activity and phenotypes in later-life environments (A. Bird, 2007; Feinberg, 2007; Jaenisch & Bird, 2003). Conceptualizing this process within a signalling framework has direct application not only to the study of human disease, but also, to the understanding of evolutionary developmental processes that directly impact phenotypes upon which selection acts (Gilbert & Epel, 2015). We present brief examples of both below.

Insect polyphenisms in evolutionary developmental biology

Insects exhibit extraordinary intraspecific diversity in appearance and behaviour (Applebaum & Heifetz, 1999; Miura, 2005), much of which is induced by environmental stimuli. One of the most conspicuous examples of developmental plasticity is the density-dependent polyphenism of swarming locusts. Both the desert locust (*Schistocerca gregaria*) and the migratory locust (*Locusta migratoria*) have two distinct adult forms: one that is gregarious in behaviour and colourful in appearance, and another that is solitary with drab colouring. These phenotypes stem from a density-dependent cue: physical contact in crowded environments early in life. Specifically, tactile stimulation on the legs of the locust nymph triggers development of the gregarious adult form (Simpson *et al.*, 2001). Although there is an extensive literature documenting the behavioural triggers, ecological factors, and pheromonal mechanisms that influence locust polyphenisms and the extent to which this developmental plasticity is adaptive (Pener, 1991; Pener & Yerushalmi, 1998), the molecular mechanisms have more recently gained prominent interest (Pener & Simpson, 2009; Simpson, Sword & Lo, 2011).

Researchers have identified gene sequences in *L. migratoria* that encode DNA methyltransferases (DNMT), a family of enzymes that catalyse the DNA methylation reaction (Robinson *et al.*, 2011). These DNMTs are differentially expressed between the solitary and gregarious phases in both *L. migratoria* (Robinson *et al.*, 2016) and *S. gregaria* (Boerjan *et al.*, 2011), suggesting that differences in early environment lead to differences in the methylation control machinery. Such differences in DNMT expression can lead to changes in the epigenome, which in turn can alter gene expression and regulate developmental plasticity. Indeed, desert and migratory locusts have relatively high levels of DNA methylation compared to many other invertebrates (Falckenhayn *et al.*, 2013), and differential methylation has been identified in over 90 genes and non-coding transposable elements associated with adult phenotypes that reflect early-life crowding conditions (Wang *et al.*, 2014; Robinson *et al.*, 2016). The dramatic differences in behaviour and morphology of locusts due to crowding conditions, despite the identical nucleotide sequence in their DNA, provides a compelling example of how epigenetic signals encoded early in life may alter adult phenotypes. Furthermore, because the locust polyphenisms are stable and involve modifications in DNA methylation, this system demonstrates how an epigenetic molecular link can transmit messages over the course of development to induce phenotypic differences later in life; an information-transmission process with parallels to communication in a potentially noisy signal system.

Poor nutrition, thrifty metabolism, and human health

In studies of human health, the field of DOHaD documents plasticity in metabolic phenotype in response to early-life nutrition (Gillman, 2005, 2010; Gluckman & Hanson, 2006;

Godfrey, Gluckman & Hanson, 2010). A central hypothesis of DOHaD is that environmental exposures during sensitive periods of development (e.g. the *in utero* period, early infancy, and the pubertal transition) have a greater impact on adult phenotype than those occurring at other times (Gluckman *et al.*, 2005a; Barouki *et al.*, 2012). This hypothesis originated from David Barker's observations of higher ischaemic heart disease mortality among persons who were small as infants – presumably due to poor intrauterine nutrition (Barker *et al.*, 1989). Subsequently, analyses of data from the Dutch Winter Famine of 1944 revealed associations between periconceptional exposure to famine and poor metabolic health in adulthood in a trimester-specific manner (Ravelli *et al.*, 1998; Roseboom *et al.*, 2001; Painter, Roseboom & Bleker, 2005).

More recent work with the Dutch Winter Famine cohort identified DNA methylation as a mechanism linking prenatal famine exposure to poor metabolic health. Heijmans *et al.* (2008) found that adults exposed to famine around the time of conception had lower methylation of insulin-like growth factor 2 (*IGF2*), a maternally imprinted gene that regulates intrauterine growth (Barlow & Bartolomei, 2007), as compared to same-sex siblings not exposed to famine. In a follow up study, Tobi *et al*. (2009) identified six additional loci involved in regulation of growth and metabolism that were differentially methylated in adults prenatally exposed to famine and their non-exposed siblings. These results provide stronger evidence that early nutrition influences DNA methylation, but because genes may be either hyper- or hypomethylated; the potential adaptive function of these alterations as an epigenetic signal warrants additional investigation (Tobi *et al.*, 2009).

Collectively, these examples from insect and human literatures highlight three important facets of developmental plasticity: (1) environmental exposures may affect phenotypes, possibly *via* epigenetic mechanisms, after a long delay, (2) the same environmental stimuli may have different effects on phenotype depending on the timing of the exposure, and (3) environmental stability affects the likelihood of a mismatch between an organism's phenotype in the predicted *versus* actual environment – a central concept to DOHaD (Godfrey *et al.*, 2007) and a determinant of Darwinian fitness (Gluckman, Hanson & Beedle, 2007; Frankenhuis & Del Giudice, 2012). We suggest below that use of a signalling theory framework illustrates how one specific epigenetic mechanism, DNA methylation, facilitates developmental plasticity.

SIGNAL SYSTEMS AND SIGNALLING THEORY

Here, we describe key aspects of a signal system, which includes the signal itself, reliability of signal transmission, and the receiver response, and relate them to epigenetic signalling. Ultimately, we lay the groundwork for Section IV, which relates signalling theory to conceptual frameworks within evolutionary developmental biology and DOHaD.

Signal systems are a key aspect of information theory, which focuses on quantification of information; by contrast, signalling theory is a sub-discipline of evolutionary biology concerned with the adaptive value of a signal, and signal detection theory is a statistical application that deals with prediction based on posterior distributionsl (Getty, 2014). Despite the subtle differences among the foci of these bodies of theory, they share interest in elucidating how signal systems are influenced by internal and external factors and assessing the

consequences of errors in signal systems. Note that throughout this paper we discuss random variability as types of process errors and signal system noise, both of which are terms that describe the role of stochasticity in signalling and biological systems. Here, we integrate concepts from each of these fields to discuss signal systems as they apply to an epigenetic signal system.

Signals and information

What is a signal?

A signal is a structure, an energetic state, or an action that transmits information in order to reduce the receiver's uncertainty about some state of nature (Hartley, 1928; Shannon, 1948, 1949; Maynard Smith & Harper, 2003; Schneider, 2014). Evolutionary biologists distinguish between signals, which evolved because they change the behaviour of receivers in ways that benefit the signaller, and cues, which benefit only the receiver (Maynard Smith & Harper, 2003; Bradbury & Vehrencamp, 2011). In our conceptualization of epigenetically mediated developmental plasticity, the signal of food scarcity, representing the organism's experience of a depauperate environment, would be considered a cue. The epigenetic signal from early to later life of an individual is a true signal if the developmental response is adaptive later in life. As with hormonal signals, the epigenetic signal benefits the sender because of the response of the receiver, which happens to be the same individual. Having noted this distinction between signals and cues, we will adopt the terminology of physical sciences and engineering and, henceforth, refer to both as signals in this review.

Information theory approaches to signalling focus on the information content of signals, measured as uncertainty reduction (Hartley, 1928; Shannon, 1948, 1949; Adami, 2004; Schneider, 2014). Evolutionary biological approaches to signalling focus on the value of information, measured by how much it can improve fitness (Dall *et al.*, 2005; Getty, 2014; Pike, McNamara & Houston, 2016). Getty (2014) illustrates with the following simple example. In a penny-matching game, the uncertainty in the outcome of a flipped penny is one bit. The value of knowing the outcome and improving the probability of winning the penny from ½ to 1 is half a penny. In the classic story *The Lady or the Tiger* (Stockton, 1882), where a man has a choice of two doors, behind one door is the lady and life and behind the other is the tiger and death, the uncertainty is the same as for the penny-matching game (one bit) but the value of knowing which door leads to the tiger is much greater than the value of half a penny. Accordingly, in signalling theory, the same quantity of information can differ considerably in value. This has important ramifications for an organism's response to environmental signals, given that the function of adaptive developmental plasticity is to improve health and/or fitness. This concept of responsiveness to signals is discussed in greater detail in Section III.4.

Quantifying epigenetic information

Mechanics of DNA methylation

We focus on DNA methylation as a mitotically heritable signal that transmits information about the environment early in life to affect an organism's phenotype later in life. We recognize that histone modifications and small non-coding RNAs also influence gene

expression without changing the nucleotide sequence, but we will not address those mechanisms here.

In vertebrates, DNA methylation is the covalent addition of a methyl group to the fifth carbon of the pyrimidine ring of a cytosine base that belongs to a cytosine–phosphate–guanine (CpG) dinucleotide pair (Razin & Riggs, 1980). Details of the DNA methylation reaction are shown in Figure. 2.1.

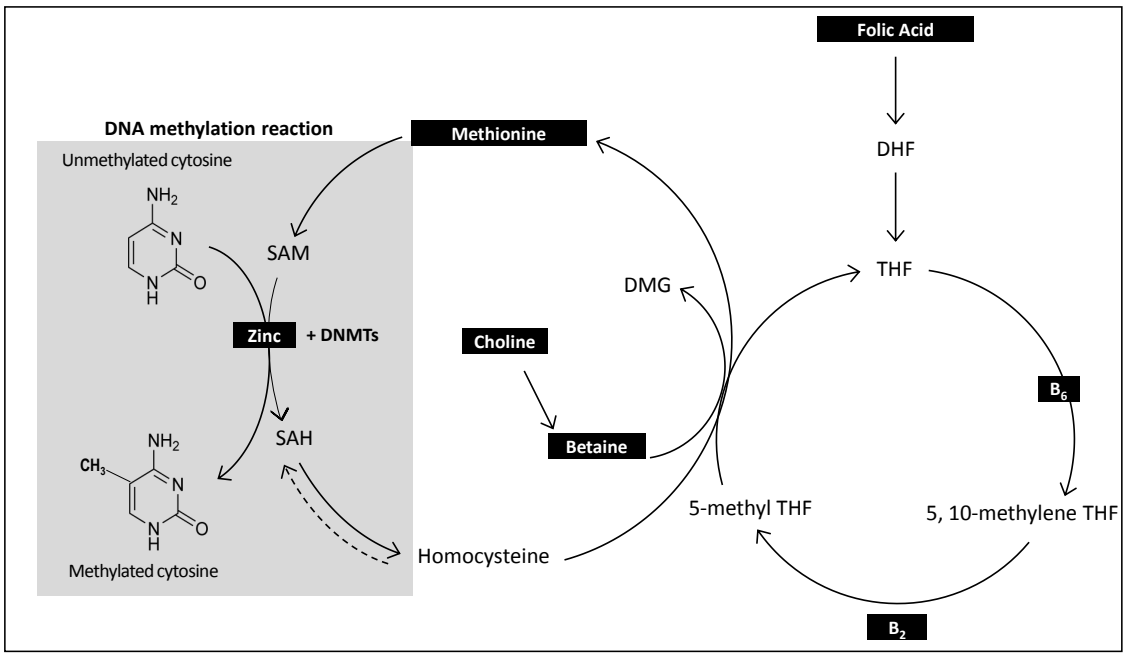

Figure 2.1. The DNA methylation reaction and one-carbon metabolism. Folic acid enters one-carbon metabolism as dihydrofolate (DHF), which is reduced to tetrahydrofolate (THF), which is converted to 5,10-methylene THF in a reaction catalysed by vitamin B6 and serine hydroxyl-methyltransferase. Vitamin B2, precursor to flavin adenine dinucleotide, is a cofactor to methylenetetrahydrofolate reductase in the conversion of 5,10-methylene THF to 5-methyl THF. Vitamin B12 is a precursor to methionine synthase, which is involved in the production of methionine and dimethylglycine (DMG) from homocysteine and betaine. Zinc is a cofactor to the DNA methyltransferases (DNMTs) in the transfer of the methyl group from S-adenosylmethionine (SAM) to the 5th carbon of cytosine. Demethylated SAM becomes S-adenosylhomocysteine (SAH), which is subsequently hydrolysed to homocysteine by adenosylhomocysteinase. Homocysteine can be recycled back to methionine with adequate methyl-donor (folate and choline) and methylation cofactor (vitamin B12, vitamin B6, vitamin B2, and zinc) micronutrients. Adapted with permission from Anderson *et al*. (2012).

DNA methylation regulates gene expression by two primary mechanisms (Klose & Bird, 2006; Li & Bird, 2007; Bogdanović & Veenstra, 2009). First, methylation may physically interrupt protein–DNA interactions by blocking transcription factors from binding to the nucleotide sequence (Watt & Molloy, 1988; Campanero, Armstrong & Flemington, 2000). Second, methylated CpG sites preferentially recruit protein complexes, which may alter chromatin structure and modify transcription (Nan *et al.*, 1998; Weaver *et al.*, 2014).

The mechanisms by which DNA methylation is established and its role in gene regulation were first proposed in 1975 (Holliday & Pugh, 1975; Riggs, 1975) and have since been a topic of great interest in biology. In brief, the majority of mammalian *de novo* CpG methylation occurs during early development following two genome-wide demethylation events, and subsequent re-establishment of methylation marks that depend on both genetic instructions and environmental conditions (Reik, Dean & Walter, 2001; Faulk & Dolinoy, 2011). The DNA methylation reaction is catalysed by a family of proteins known as DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B, all of which interact with DNA, RNA, and other proteins preferentially to methylate certain CpG regions while leaving other regions unmethylated (Goll & Bestor, 2005; Klose & Bird, 2006). The addition of methyl groups by DNMTs can enhance, reduce, or maintain gene expression in response to environmental factors (Bird, 2002; Jaenisch & Bird, 2003). Methylation can also be removed from CpG sites *via* ten-eleven translocation (TET) enzymes (Kohli & Zhang, 2013). Coupled with DNMT activity, TET enzymes allow for more dynamic coordination of epigenetically controlled gene expression.

DNA methylation as a signal

We consider an epigenetic signal as a cluster of CpG sites in which DNA methylation corresponds to bits of information. Clusters of CpG sites that occur at high densities, often in the promoter region of genes, are referred to as CpG islands (Bird *et al.*, 1985; Illingworth & Bird, 2009) and changes in DNA methylation at CpG islands have potential to alter chromatin structure and influence gene expression (Bird, 1986; Deaton & Bird, 2011). Flanking either side of CpG islands are the slightly less GC-rich CpG island shores (Irizarry *et al.*, 2009), followed by even more distally located CpG island shelves (Bibikova *et al.*, 2011), which can be variably methylated and may also be associated with developmental differences in gene expression. Considering a genomic region that includes CpG clusters as a signal, we view each dinucleotide pair as a potential CpG site, and assume that all CpG sites can be in one of two states, methylated or unmethylated. Therefore, each CpG site may be viewed as an information-storage position that can take on values of 0 (unmethylated) or 1 (methylated). Collectively, a CpG cluster represents a binary sequence, which is similar in structure to the lines of bits in basic computer code, and that can influence gene expression.

The quantity of information in an epigenetic signal varies. In a section of DNA of fixed length, where n = number of nucleotides, there are 0 to $n/2$ CpG dinucleotide pairs. Considering that methylation status of any particular CpG site is binary, a single CpG site may function as an on/off switch *via* direct blocking or dim gene expression by reducing the probability that a transcription factor binds to the DNA. The presence of multiple CpG sites in the same section of DNA may allow for multiple combinations of ‘on’ and ‘off’ that together function like bytes of information in computer code. As the number of CpG sites and the quantity of information

within an epigenetic signal increases, the number of outcome combinations increases exponentially, which may contribute to the precision of gene regulation in the same way that the arithmetic precision of digital computers can increase with the byte length of the central processing unit (CPU). In a biological context, DNA methylation of a CpG site in a single cell may physically block a transcription factor from accessing the domain within which the CpG site is located, thereby effectively turning off transcription in that specific cell. Conversely, removal of the methyl group from that same CpG site would allow the transcription factor to bind, and the cell could proceed with RNA transcription. The quantity of information based on methylation status of multiple cells within a tissue, even at a single CpG site, is measured as the average of binary methylation status (yes/no) across all cells. The potential phenotypic variation that results from an epigenetic signal increases as we consider multicellular tissue organization, multiple CpG sites, and higher-order interactions of DNA with methyl-sensitive proteins and chromatin folding mechanisms that can affect transcription across a continuum.

The value of a single bit *versus* combinations of bits of information in an epigenetic signal depends on how that information influences the organism's phenotypic response, and the extent to which that response is expected to enhance fitness. In the following sections, we expand upon this idea by reviewing the parts of a signal system, concepts of signal reliability, and the adaptive value of the receiver response in the form of developmental plasticity. However, before we continue, it is important to acknowledge some simplifying assumptions that we make in order to focus on DNA methylation as an example of an epigenetic signal. Specifically, the role of DNA methylation in relation to gene expression is complex and context dependent (Jones, 2012), but we will proceed with the generalization that higher promoter CpG

methylation results in lower expression of the corresponding gene, whereas lower CpG methylation enhances expression (Li & Bird, 2007; Siegfried & Simon, 2010). For simplicity, we will not discuss the effects of transitional methylation chemical configurations, like hydroxymethylcytosine (Tahiliani *et al.*, 2009), which often occur around transcriptional start sites and enhancers and may be important for determining phenotypes (Yu *et al.*, 2012). We also recognize but do not formally address the fact that there may be correlations in the methylation states of neighbouring and/or functionally related CpG sites, which violates an assumption of information theory that each storage position is independent of other storage positions. However, accounting for the complex interrelations among CpG sites is beyond the scope of this review.

Basic signal system design

In his classic paper, Shannon (1948) developed a schematic for a general communication system, which we have modified slightly to enhance clarity (Figure 2.2).

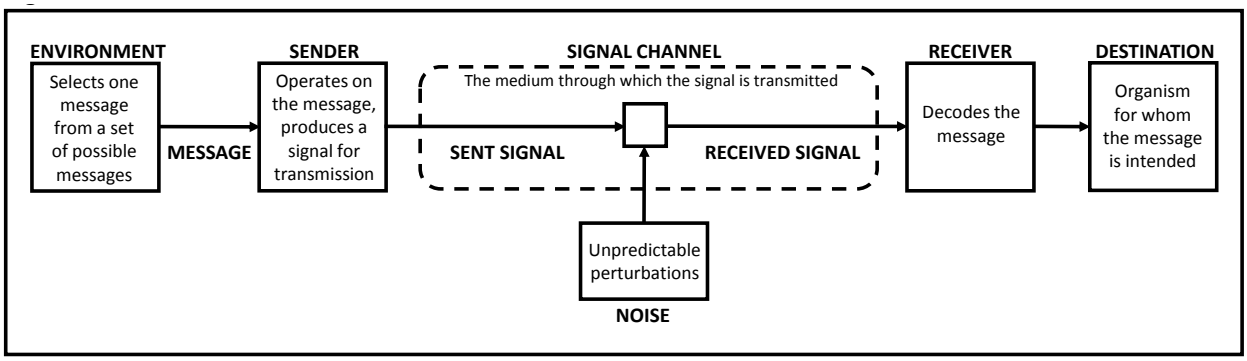

Figure 2.2. Shannon's original system design. Adapted from Shannon (1949).

In Figure 2.3, we adapt Shannon's representation to illustrate our conceptualization of epigenetic mediation of adaptive developmental plasticity (Bateson *et al.*, 2004; Gluckman, Hanson & Spencer, 2005*b*) as a noisy communication system.

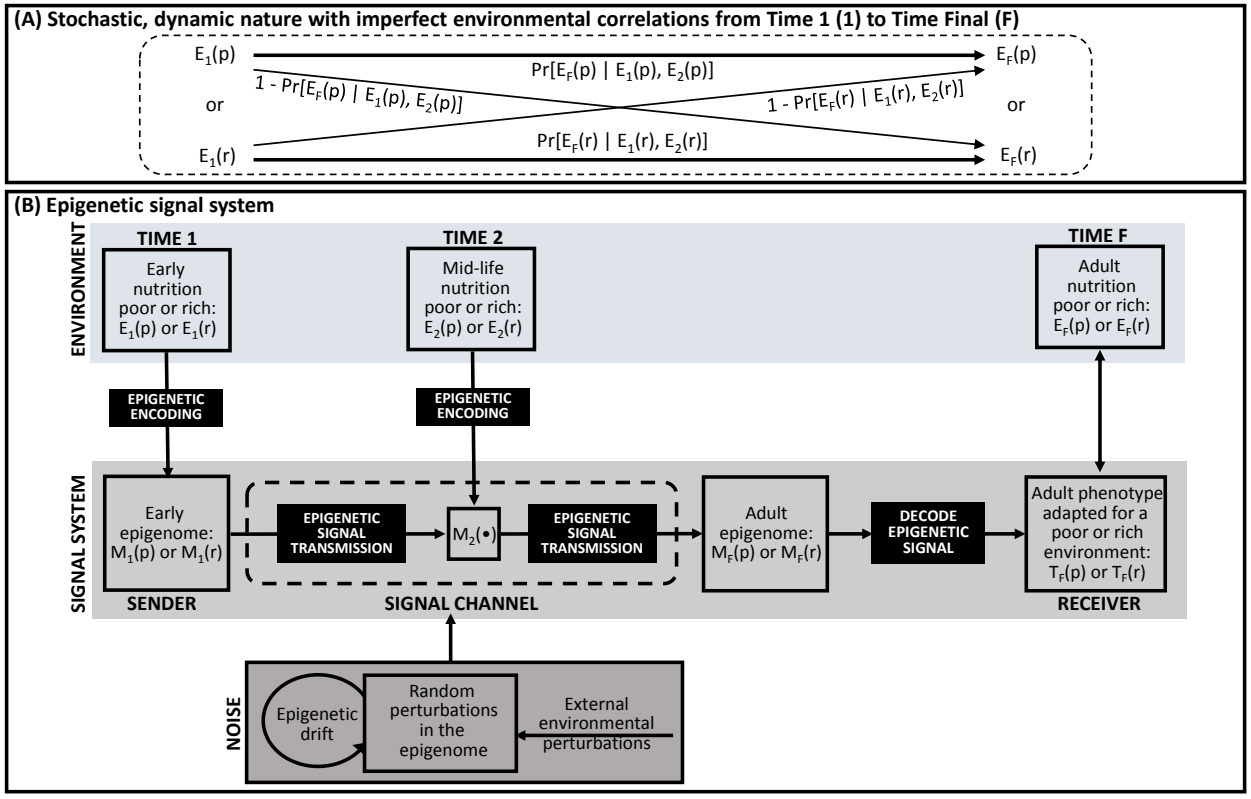

Figure 2.3. (A) A dynamic environment over time. E_1 and E_F represent the early and later environments, respectively, which may be either nutrient poor (p) or rich (r). PR […] represents conditional probability of a match (e.g. poor early-life environment/poor later-life environment) or mismatch (e.g. poor early-life environment/rich later-life environment). (B) An epigenetic signal system where environmental factors impact DNA methylation at multiple points in time across an organism's life course (M_1, M_2, M_F) depending on a nutrient-poor (p) or -rich (r) environment. Adult phenotype is represented as $T_F(p)$ or $T_F(r)$ reflecting the phenotypic response. Epigenetic drift and environmental perturbations (i.e. deflection) both contribute stochastic variability, or process noise, to the signal.

An organism's response to environmental stimuli *via* epigenetic mechanisms fit neatly into this signal system paradigm. As a signal sender, an organism encodes information from the environment, and transmits it over the course of development as DNA methylation marks that

are analogous to bits in a byte. At a future time point, the organism is the signal receiver, who decodes the signal and uses the information to guide its phenotypic response (Figure 2.3B).

Signal reliability

The motivation to understand signal reliability is to elucidate how signals permit communication (Hasson, 1994, 1997; Hurd & Enquist, 2005; Wiley, 2006). When considering signal reliability, we focus on the signal sender (the organism at present), properties of the signal, and a signal receiver (the organism in the future) (Bradbury & Vehrencamp, 2011). Successful communication occurs when the signal is efficacious enough to reach the receiver (efficacy) and the information is meaningful to the receiver (content) (Guilford & Dawkins, 1993). Signal efficacy is the capacity of the signal to transmit from the sender and be detected by receiver (Guilford & Dawkins, 1993), and depends on the signal's physical structure as well as properties of the signal channel (e.g. distance between sender and receiver and background noise) that influence signal detectability and discriminability. On the other hand, signal content refers to the actual message and the ability of the receiver to understand the message in order to reduce uncertainty or improve prediction.

Signal efficacy

Efficacy in biological communication depends on the signal-to-noise ratio (Beal, 2015). There are three aspects to consider in regard to signal efficacy: (1) the signal's structure and

intensity, (2) internal system errors (noise), and (3) external errors (background noise) (Guilford & Dawkins, 1991; Endler, 1992).

To start, we can use an example of people talking through a string and tin-can telephone to conceptualize signal efficacy. Person A speaks "Watch out below," into a tin can. The auditory signal arrives at the tin can of the receiver, Person B, and the message is decoded. A louder spoken message improves efficacy by increasing the amplitude of the sound waves. When thinking of signal efficacy, for example, in regard to an epigenetic signal communicating information about the organism's nutritional environment, we propose that, at an initial time point, an organism (the signal sender) encodes information about its current environment [$E_1(\bullet)$ in Figure 2.3B] as DNA methylation marks on CpG sites [$M_1(\bullet)$] that are transmitted to the future. At a later time point, the organism (now the signal receiver) reads the methylation marks [$M_F(\bullet)$] and translates them into a phenotype [$T_F(\bullet)$], which might or might not be a good match for the adult environment [$E_F(\bullet)$]. For example, in a nutrient-poor early-life environment such as that caused by famine [$E_1(p)$], the young organism encodes the information about its current environment by decreasing methylation of gene regions involved in growth and metabolism [$M_1(p)$]. Then, the message is transmitted to later in life with imperfect fidelity due to internal process errors, as discussed in the next section. The older organism decodes the epigenetic signal [$M_F(p)$], and develops a metabolically efficient ('thrifty') phenotype [$T_F(p)$] in anticipation of a nutrient-poor environment [$E_F(p)$]. In the next two sections, we describe the process of transmitting epigenetic signals, specifically focusing on the potential impact of internal and external noise in the epigenetic communication channel.

Internal process errors: epigenetic fidelity

Internal process errors in a signal system reduce signal fidelity. In the tin-can telephone example, the length of the string affects the integrity of the auditory message. A shorter string will yield lower attenuation of sound waves than a longer string, resulting in a more-conserved (e.g. higher fidelity) message decoded by the signal receiver at the later life stage. Similarly, DNA methylation is subject to internal copy mechanism errors that act as noise, ultimately degrading fidelity of the epigenetic signal during transmission. Once established, DNA methylation marks are clonally inherited as part of DNA replication during each cell division (Bestor & Tycko, 1996; Chen & Riggs, 2005). The newly formed DNA is asymmetrical and hemi-methylated, so reliable propagation of the epigenetic signal requires restoration of complementary methylation. The protein NP95, also known as UHRF1 (ubiquitin-like with plant homeodomain [PHD] and ring finger domains), has an affinity for hemi-methylated DNA and recruits DNMT1 to restore complementary methylation (Ooi & Bestor, 2008). This process of methylation maintenance results in faithful transmission of epigenetic signals with over 95% accuracy in both theoretical (Pfeifer *et al.*, 1990; Riggs & Xiong, 2004) and empirical models (Laird *et al.*, 2004). However, fidelity of methylation maintenance is not guaranteed and random errors, known as epigenetic drift, can occur (Fraga *et al.*, 2005; Wong, Gottesman & Petronis, 2005). Twin studies have shown that, despite shared genetics and prenatal environment, random errors in epigenetic signals arise during the aging process and contribute to phenotypic divergence between individuals over time (Fraga *et al.*, 2005; Martin, 2005; Fraga & Esteller, 2007). The process of epigenetic drift is caused by the failure to recapitulate DNA methylation faithfully during cell division, and emphasizes the role of stochasticity in modifying

the phenotypes of organisms that transmit epigenetic signals across many cell divisions and over long time periods (Wong *et al.*, 2005; Shibata, 2009). The extent to which epigenetic signals are subject to drift, and how much internal error affects signal reliability are likely to have important implications for developmental trajectories and adult onset diseases, as well as for the evolution of predictive adaptive responses (PAR).

Notably, when considering epigenetic signal efficacy, it is important to keep in mind that genetics also plays a role. Genomic sequence variants may limit the available CpG sites for information storage, and background genetic variation may alter the form and function of downstream proteins responsible for DNA methylation maintenance (Bjornsson, Fallin & Feinberg, 2004). For example, the degree of global methylation stability over time (which likely includes changes due to drift) is more similar within than between families, suggesting a genetic basis for the accumulation of epigenetic errors over time (Bjornsson *et al.*, 2008). Genetic variation could thus hypothetically alter epigenetic drift trajectories to affect signal reliability by contributing noise to the system.

External process errors: environmental perturbations

Epigenetic signals are also subject to external sources of error. Specifically, environmental perturbations occurring between the initial developmental time point of interest early in life and the later time point when phenotype is assessed, can introduce noise and exacerbate random errors in the epigenetic signal system [forcing differences between $M_1(\bullet)$ and $M_F(\bullet)$]. In our tin-can telephone example, a spoken signal will be clearer in a quiet room,

where sound waves are not perturbed by ambient noise, than in a room full of conversing people.

To illustrate the concept of external process errors, we present a few examples from the human literature. A study of monozygotic twins found greater variability in the epigenomes of adult than children twin pairs, as well as greater differences between the epigenomes of adult twins who grew up in different than in similar environments (Fraga *et al.*, 2005). The latter finding suggests that, in addition to internal noise (e.g. imperfect replication of methylation marks as part of multiple cell divisions), signal efficacy is also susceptible to external noise (e.g. environmental perturbations like toxicants, hormones, nutrition etc.). Early-life exposure to environmental toxicants can influence DNA methylation and ultimately, increase risk of adult disease (Dolinoy, Huang & Jirtle, 2007*b*). Broadly speaking, when environmental factors like toxicants (Kundakovic *et al.*, 2013) or synthetic hormones (Crudo *et al.*, 2012) alter expression of DNA methylation maintenance machinery, the effect may be to alter DNA methylation patterns and perhaps also to change the rate of epigenetic drift. For instance, prenatal exposures to lead (Faulk *et al.*, 2014) and bisphenol A (BPA) (Kochmanski *et al.*, 2016) are associated with altered age-related methylation changes. Such findings indicate that a variety of external factors may compromise signal efficacy by altering the rate of epigenetic drift (Kochmanski *et al.*, 2017). However, the extent to which the environment is simply a source of external noise, rather than a force that elicits deterministic changes in the epigenome, are two separate concepts which may be difficult to disentangle empirically.

Signal content

The second part of signal reliability is the meaning of the message (Guilford & Dawkins, 1991; Maynard Smith & Harper, 2003). In our conceptualization (Figure 2.3) the meaning of the signal (i.e. the message), which is encoded as $M_F(\bullet)$, is translated into trait $T_F(\bullet)$. Above, we focused on the potential roles of internal and external noise in degrading signal reliability. Signal content may also be unreliable if either the message or the environment changes over the course of transmission. We thus divide potential threats to signal content into two categories: (1) deterministic changes to signal content [e.g. potentially adaptive, population level changes to $M_1(\bullet)$ and $M_2(\bullet)$ that result in $M_F(\bullet)$], and (2) environmental instability [e.g. probabilistic differences between $E_1(\bullet)$ and $E_F(\bullet)$, given $E_1(\bullet)$ and $E_2(\bullet)$].

Deterministic changes to signal content

During vulnerable windows of development, such as the prenatal period, infancy and puberty, the epigenome is particularly sensitive to environmental exposures (Dolinoy *et al.*, 2007*a*; Faulk & Dolinoy, 2011). Accordingly, these are prime timeframes for encoding and modifying epigenetic messages in response to the environment, allowing for potentially adaptive plasticity. The environment, $E_1(\bullet)$, at an initial time point, provides information that is encoded into the epigenome $M_1(\bullet)$, which may improve the organism's performance in its future environment, $E_F(\bullet)$; (Figure 2.3). Later in ontogeny, another environmental factor, $E_2(\bullet)$, might modify the epigenome $M_2(\bullet)$. The combination of environmental factors will each contribute (additively or multiplicatively) to the future epigenome $M_F(\bullet)$, which is then decoded as information used for a phenotypic response $T_F(\bullet)$. Because epigenetic signals transmit

through a sequence of developmental windows, environmental information entering through a later window might modify the message from an earlier window. For example, animal models indicate that maternal peri-conceptional and gestational nutrition affect offspring phenotype through epigenetic mechanisms (Waterland *et al.*, 2006; Sinclair *et al.*, 2007; Carlin, George & Reyes, 2013), but also that offspring DNA methylation patterns can remain responsive to nutrition over the life course (Cordero *et al.*, 2013), particularly during developmental life stages characterized by rapid growth or development and/or hormonal fluctuation, such as during infancy, puberty, and pregnancy. The addition or removal of DNA methylation in response to multiple environmental exposures that occur throughout development may compromise signal reliability by changing the meaning of the original message.

Changes in DNA methylation can also affect signal content by inducing changes in the underlying genetic code. First, the addition of methylation can lead to deamination of methylated cytosine into thymine, resulting in a cytosine-to-thymine point mutation (Bird, 1980), and can reduce the number of CpG sites, leaving the signal depleted of information-storage positions (Simmen, 2008). Second, removal of methylation can lead to activation of transposons, which include mobile segments of remnant viral DNA that can propagate themselves throughout the genome *via* cut-and-paste or copy-and-paste mechanisms (Finnegan, 1989; Slotkin & Martienssen, 2007). While the former reduces the storage potential of epigenetic signals due to removal of CpG sites, the latter may increase information storage potential by reseeding CpG sites. Thus, both changes in CpG site density due to nucleotide mutations or transpositions that are sensitive to methylation status, and changes in DNA methylation in response to environmental exposures, can influence epigenetic signal content.

Environmental instability

Content-related signal reliability also depends on stability of the environment. Both long transmission distances (i.e. time) and unstable environments can make future operating environments too unpredictable to anticipate adaptively. The likelihood that $E_1(\bullet)$ and $E_F(\bullet)$ are the same is based on the conditional probability of $E_F(\bullet)$, given $E_1(\bullet)$ and $E_2(\bullet)$; (Getty, 1996 and Figure 2.3A). For example, an organism in a nutrient-restricted environment encodes a DNA methylation pattern that emphasizes the need for a thrifty metabolism. Later in life, the organism receives the methylation signal and responds with gene expression that produces a thrifty metabolism. If the signal indicating a food-deprived environment from early life accurately captures the later-life food environment, then the signal's content improves the organism's performance. On the other hand, in the event of a mismatch between the food environments in early and later life, the thrifty phenotype may be maladaptive in the later-life environment (Godfrey *et al.*, 2007). Whether or not an environment changes over time is independent of the signal transmission process, but together signal reliability and environmental instability determine whether or not a mechanism can evolve that accurately anticipates a probabilistic future environment and develops the appropriate adaptive phenotype for that environment.

Receiver response: signal detection theory

Developmental plasticity is a form of receiver response, as it represents the capacity of an organism to modify its phenotype based on information about its environment previously

encoded as an epigenetic signal. In a biological signal system, an organism can either respond to or ignore a signal using a set of rules, referred to as decision criteria (Wiley, 2006; Anderson, 2015). Decisions are based on whether or not the signal improves prediction, and the probable costs and benefits associated with a response. Given the two response options (respond or reject) there are four possible outcomes that represent the match between receiver response $T_F(\bullet)$ and the environment $E_F(\bullet)$; (Figure 2.4A). These four outcomes can be mapped as a two-by-two contingency matrix: correctly respond to a signal, correctly reject noise, incorrectly respond to noise, and incorrectly reject a signal (Wiley, 2006; Anderson, 2015).

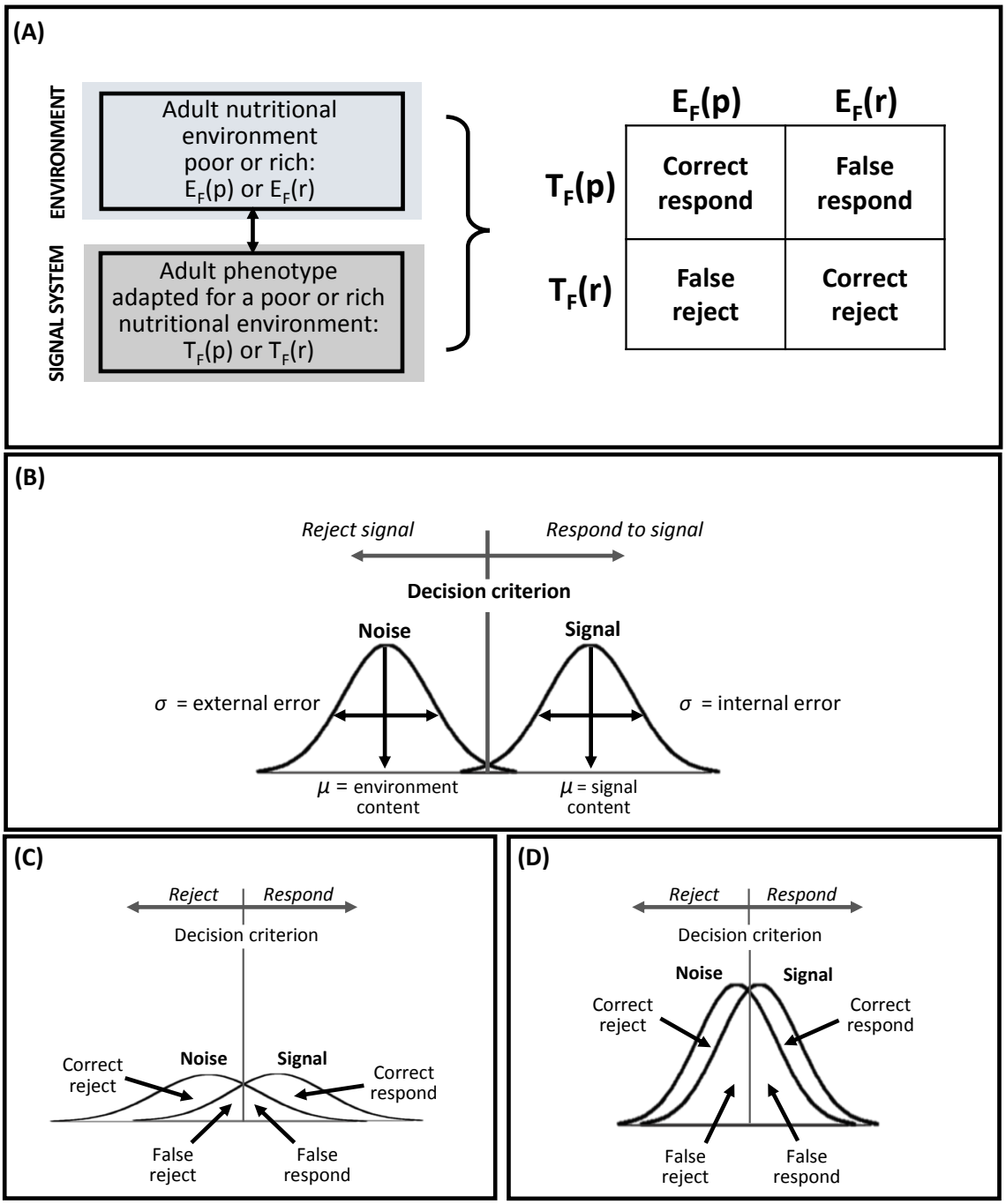

Figure 2.4. (A) A depiction of signal detection theory and determinants of a matched or mismatched response where E_1 and E_F represent the early and later environments, respectively, either of which may be nutrient poor (p) or rich (r). (B) Distribution of noise and information in a signal system, and determinants of response to a signal. (C) Scenario of reduced efficacy. (D) Scenario of modified signal content.

This contingency table can be used to assess a receiver response, where that response is a discrete phenotypic state that depends on both the reliability of an epigenetic signal and the ability of the receiver to decode the message.

As part of this assessment we can quantify two useful epidemiological measures of predictability, sensitivity and specificity. Sensitivity is the ability to respond to a signal when it is appropriate to do so, and specificity is the ability to reject a signal when it is inappropriate (Rothman, Greenland & Lash, 2008). Each cell in the contingency table is effectively an area under curves representing noise and signal above or below the decision criterion (Figure 2.4B). The phenotypic response can also be described continuously by integrating the probability distribution functions for noise and signal between the limit of the decision rule (the value of the *x*-axis intersection) and infinity in positive or negative directions. Then, comparing the areas under the curves with a signal-to-noise ratio we can generate receiver operating characteristic (ROC) curves to assess the predictive capacity of an epigenetic signal, and even identify an optimal threshold at which the signal-to-noise ratio should elicit a response (developmental plasticity). By comparing the noise and signal distributions alongside the contingency table or ROC curves, we can evaluate how epigenetic signal reliability influences prediction, and the probability with which receiver response results in a match between the phenotype and environment.

Receiver response based on reliability

Receiver response depends on signal efficacy (e.g. process errors) and content (e.g. the message). Reduced signal efficacy is synonymous with increased internal and external errors in

the signal channel and accordingly, more variance in the noise and signal distributions (Figure 2.4C). For example, the longer the time between signal establishment and reception of the signal, the greater the potential for internal errors in methylation (i.e. more epigenetic drift) and accordingly, the greater the variance in the signal distribution. Likewise, external errors caused by environmental perturbations increase the variance in the noise distribution. Ultimately, higher rates of internal and external errors lengthen the tails of the signal and noise distributions, respectively, increasing the degree of overlap between the distributions to reduce signal discriminability (Figure 2.4C).

Receiver response also depends on reliability of signal content. Here, the mean of the signal distribution represents the signal's content and mean of the noise distribution represents background noise content (e.g. any background unrelated to the signal's message). If we imagine a collection of epigenetic signals, those that have a number of methylated CpG sites closest to the signal mean and furthest away from the background noise mean will have the clearest message, and thus, are expected to influence an organism's phenotype most consistently. Factors that shift the signal mean towards the mean of the noise distribution, such as changes in DNA methylation that result from conflicting information from multiple environmental sources, increase the overlap between the two distributions (Figure 2.4D). Increasing overlap in signal and noise distributions obscures the message and reduces the probability that a receiver will benefit by acting on the message, so a positive response is increasingly likely to be a false response.

Considering together the effect of signal efficacy and content on receiver response in our thrifty phenotype example, correctly anticipating a nutrient-poor adult environment [$T_2(p)$

matched to $E_2(p)$] corresponds to a 'correct response' (Getty, 1996; Figure 2.4A). Correctly anticipating a nutrient-rich adult environment [$T_2(r)$ in $E_2(r)$] is a 'correct reject'. The two scenarios depicted in Figure 2.4C and D both decrease discriminability between signal and noise, decreasing the probability that a positive developmental response to an early signal will adaptively match the later phenotype to the later-life environment.

Receiver response based on signal value

The value of a receiver response, and by extension, the value of the signal, depends on whether a particular decision to respond and the resultant phenotype are adaptive or maladaptive in the organism's later-life environment. We can quantify the value of an organism's phenotypic response to an epigenetic signal using the fitness trade-off matrix (Figure 2.5). The fitness (ω) of a communication system for making PARs depends on the trade-offs associated with the receiver response, as well as signal reliability and environmental stability within the organism's lifetime. This means that optimization of the decision-making process based on reliability alone is not always the best strategy. Flexibility in receiver response reflects the ability of an organism to balance trade-offs between signal reliability and signal value.

Assuming that the decision criterion can adapt or evolve, an organism's responsiveness to environmental signals should vary with the costs of errors (false reject, false respond) as well as the benefits of correctly rejecting and correctly responding. When the cost of rejecting a true signal is high relative to responding to noise, then lowering the criterion for responding is optimal; a scenario we refer to as reactive plasticity (Fig. 5) (Getty, 1996).

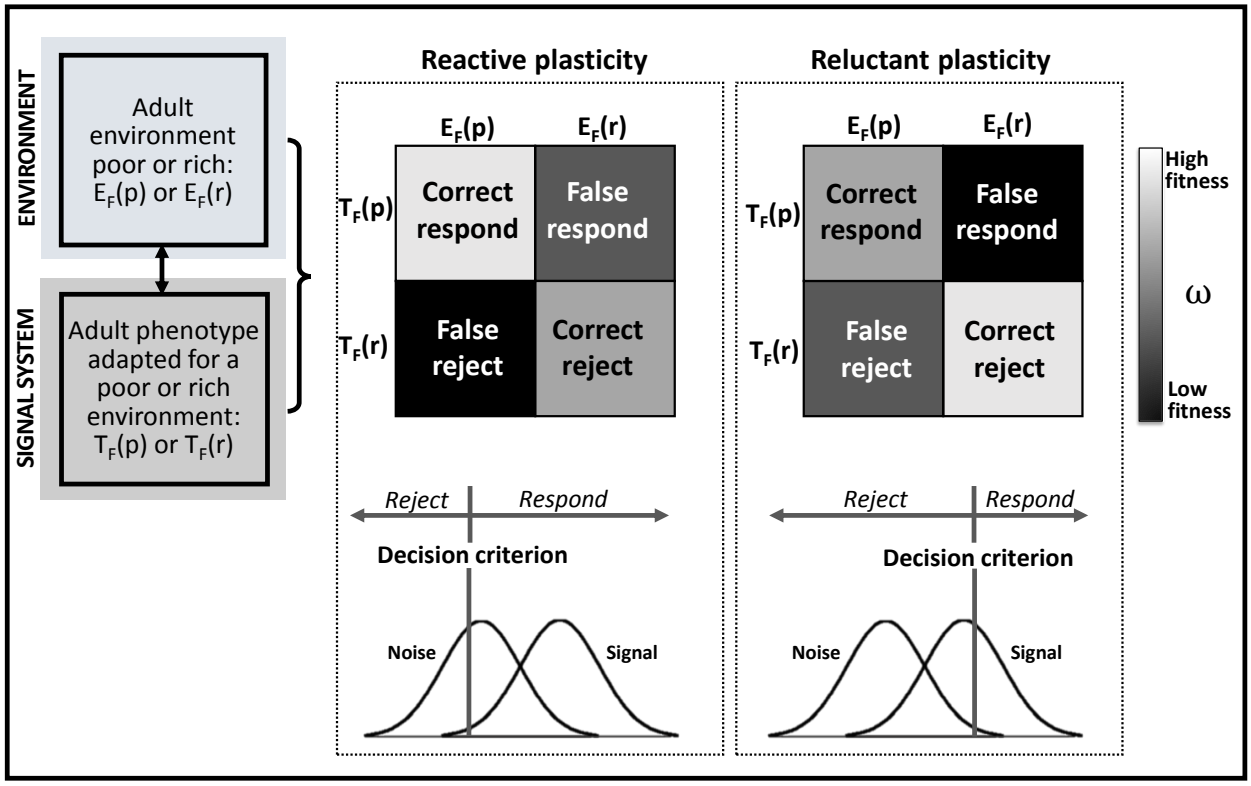

Figure 2.5. Alterations in decision rules based on signal value. E_1 and E_F represent the early and later environments, respectively, either of which may be nutrient poor (p) or rich (r). Fitness (ω) depends on the performance of a phenotype in its present environment. Organisms are expected to be more likely to show a phenotypic response when the fitness costs of a false rejection are highest and they are less likely to respond when the fitness costs of a false response are highest; thus the value of the information in the signal influences receiver response.

For example, an organism born in a nutrient-limited environment is unlikely to survive unless growth is restricted. Alternatively, although growth restriction in a nutrient-abundant environment is not ideal and may lead to metabolic disease, it is not as costly as starvation. Here, the decision criterion would shift left such that the organism is more likely to respond by growth restriction, even if the epigenetic signal is weak.

Alternatively, the decision criterion might shift right, limiting responses to signals that indicate only the most extreme environments (Figure 2.5). When responding to noise is more costly than rejecting a signal, an extremely high response criterion is appropriate. Conspicuous epigenetic signals, like genome-wide demethylation induced by extreme environmental stressors, could induce genetic and phenotypic variation by releasing transposable elements from their repressed state (McClintock, 1984; Hunter *et al.*, 2014). More biological variation *via* transpositions and other mutations could be an adaptive process, possibly enabling a small portion of the population to survive (Shapiro, 2017). However, the risks associated with genome-wide destabilization are also extremely high given that genomes are the product of a long evolutionary history. Such high-stake situations should favour a very high response criterion in which organisms overcompensate by rejecting noise at the expense of potentially rejecting information; a strategy that can be thought of as 'reluctant plasticity'.

MERGING HYPOTHESES REGARDING DEVELOPMENTAL PLASTICITY WITH LIFE-COURSE MODELS

There are a number of hypotheses pertaining to biological pathways underlying developmental plasticity and the potential for a match (or mismatch) between an organism's phenotype and its environment: the predictive adaptive response hypothesis (Bateson *et al*., 2004; Gluckman *et al*., 2005*b*), the thrifty phenotype hypothesis (Hales & Barker, 2001), and the DOHaD hypothesis (Gillman, 2005). Each of these shares the view that environmental stimuli during early development can alter an organism's later-life phenotypes. In this section, we briefly describe theoretical models from life-course epidemiology, which is a methodological framework used to conceptualize and test biological pathways linking early-life experiences and

exposures to health throughout the life span. Although concepts from this field have only been systematically applied in human studies, they are relevant to developmental plasticity observed in other organisms studied by evolutionary developmental biologists.

There are two broad categories of life-course models: critical period models and risk accumulation models (Kuh *et al.*, 2003). Generally speaking, critical period models posit that experiences during sensitive developmental windows early in life lead to permanent changes in phenotype that are not substantially altered by subsequent experiences. Metaphorically, there is a distinct window of responsiveness to environmental information. After it closes, development is canalized. In Figure 2.3, midlife signals [$M_2(\bullet)$] are ignored. This model aligns with the polyphenism observed in the desert locust described in Section II.1. Indeed, after establishment of methylation marks during the juvenile stage, the morphological and behavioural phenotypes of the locust are established for life. On the other hand, risk accumulation models suggest that the effects of environmental factors or risk exposures accumulate gradually, and may interact (e.g. later exposures may exacerbate or mitigate health effects of previous exposures) over an organism's life span (the window stays open and midlife signals [$M_2(\bullet)$] significantly modify the early signal [$M_1(\cdot)$]). An example of this is prenatal BPA exposure and methyl-donor supplementation in Agouti mice; here nutrient supplementation mitigates the hypomethylating effects of BPA exposure on the A^{vy} region, indicating an interaction between BPA exposure and nutrient supplementation (Dolinoy, Huang, et al., 2007). Although nutrient supplementation attenuates the adverse impact of BPA, the reverse is also possible; in other cases subsequent environmental factors exacerbate adverse effects of earlier exposures (Hahn-Townsend *et al.*, 2016), potentially *via* epigenetic modifications.

Assessing epigenetically mediated developmental plasticity within both a signalling system and life course epidemiological framework is valuable for two reasons. First, a clear conceptualization of the temporal relations among exposures, mediators, and outcomes of interest using life-course epidemiology models will directly inform study design. Second, superimposing signalling system concepts onto life-course models can help to identify process error in signal transmission and formulate analytical strategies to parse out the impact of different types of variation (e.g. stochastic *versus* deterministic) on relationships among an organism's early environment, DNA methylation marks, and the organism's future environment and phenotype.

When considering an adaptive epigenetic signalling system that follows critical period models, an epigenetic signal is encoded during gestation or infancy, and that message directly affects the organism's future phenotype. In an error-free signal system, a study testing this hypothesis would require: (1) assessment of the environmental factor during an initial developmental period, (2) assessment of the epigenetic signal at any point in time following the developmental period of interest, assuming it remains stable after initial establishment, (3) assessment of the phenotype in its later-life environment, and (4) assessment of the correlation between early and late environments. An example is methylation of imprinted genes, like *IGF2*, which is established in gametes prior to conception and remains unchanged throughout development (Barlow & Bartolomei, 2007). Accordingly, an analytical strategy could be a standard mediation analysis where the nutritional exposure is the independent variable, *IGF2* methylation is the mediator, and adult phenotype is the dependent variable. If DNA methylation is the sole mechanism linking early nutrition to future phenotype, then inclusion of

IGF2 methylation in the model as a mediator would wholly attenuate the regression β-estimate for early nutrition. Although the simplicity of critical period models is appealing, it is likely that methylation marks, including those on imprinted genes, are subject to process errors in the form of epigenetic drift, which may contribute to endogenous plasticity (Stamps, 2016), and/or deflection, which incorporates external perturbations as recently shown in a mouse model (Kochmanski *et al.*, 2016).

Risk accumulation models suggest that both DNA methylation signals, and later-life phenotypes, are affected by the accumulation of, and interactions among, environmental stimuli across development. One example of this model is that of prenatal BPA exposure and methyl-donor supplementation in Agouti mice described above (Dolinoy, Huang, et al., 2007). When designing a study to test this model, one might be interested either in examining the independent effect of exposures during specific developmental periods or quantifying the cumulative effects on phenotype of exposures throughout the life course. Although data collection and study design for both are similar, the appropriate analytical strategy differs. We have described modelling techniques in greater detail in Laubach *et al*. (2017). In brief, testing accumulation of risk models requires appropriate partitioning of phenotypic variances due to deterministic and stochastic processes, as both are hypothesized to affect DNA methylation over time. This may be done using mediation analysis to isolate direct effects of specific developmental stages, linear mixed models to capture both deterministic changes to DNA methylation *via* main effects and stochastic individual variability *via* empirical best linear unbiased predictors (EBLUPs).

CONCLUSIONS

(1) Epigenetic marks, such as DNA methylation, may serve as mechanistic links between environmental factors that organisms experience during development and their resultant phenotype, thereby enabling adaptive developmental plasticity – a phenomenon that is well recognized in both evolutionary developmental biology and human health.

(2) We used a basic signal system design to conceptualize ways in which DNA methylation can act as a signal relaying information about an organism's early-life environment to its future self in order to improve fit between future phenotype and environment. Considering epigenetic marks as signals provides a framework within which to identify potential sources of external and internal information-transmission errors, develop appropriate study designs based on biological plausibility, and parameterize statistical models to reflect biological processes accurately.

(3) The evolutionary maintenance of adaptive developmental plasticity *via* epigenetic signalling represents a proximal mechanism for organisms with fixed genomes to respond adaptively to environmental stimuli within their lifetimes. This has implications for human health and for evolutionary theory. We hope that this paper facilitates an increasingly open and interdisciplinary approach to studying epigenetically mediated developmental plasticity that will improve understanding of both evolutionary developmental biology and determinants of human health and disease.

(4) Given the complexities of an epigenetic signal system where the signal is subject to change over time, use of appropriate statistical techniques to capture this nuance is critical. An intuitive frequentist approach would be a standard mediation analysis where the predictor is the

environmental factor of interest during early life, the outcome is phenotype at a later life stage, and the mediator is change in DNA methylation over time, parameterized as trajectories. This strategy is an improvement upon traditional mixed-model approaches (or, simply averaging DNA methylation over time), as it considers the potential influence of temporal variability in DNA methylation. However, a limitation of this approach is that the trajectories do not distinguish between deterministic and stochastic modifications of methylation marks, and thus may yield less-reliable estimates of association.

(5) Looking forward, we propose use of a Bayesian decision-theoretic approach to model phenotypic plasticity over the course of development. One could model prospective changes in DNA methylation in response to a series of environmental factors that are experienced over the course of ontogeny, in which the DNA methylation at a given point in time is conditional on methylation at a previous life stage. As an organism develops, environmental factors can have deterministic effects on the DNA methylation mark of interest, which represents an 'update' from the previous methylation state. In this example, the posterior distribution for the estimate representing the relationship between the environmental factor of interest and DNA methylation at the earlier life stage would serve as the prior for the estimate of association representing the relationship between the environmental factor later in life and the methylation mark (Stamps & Frankenhuis, 2016). Using this type of model, it is possible to assess how a series of previous environmental experiences modify the epigenetic signal. Ultimately, this may enable more accurate statistical modelling of the relationships among early environment, DNA methylation, future environment and future phenotype.

(6) Going a step further, Bayesian stochastic process models may be used to partition variance further due to deterministic and stochastic influences on the relationship between an epigenetic signal and the resultant phenotype. Accounting for stochastic changes (e.g. drift and/or deflection) as well as deterministic ones is important, as they too may alter the epigenetic signal. Use of stochastic process models would allow us not only to account appropriately for deterministic variability in DNA methylation over time, but also, such models partition variance in change in DNA methylation due to stochastic variability, which encompasses both external and internal process errors (Bolker, 2008). These models have potential to improve accuracy of statistical methods used to capture the true relationships among early life exposures, epigenetic mechanisms, and future phenotype. In evolutionary developmental biology, such models would provide a better quantification of the phenotypic variation upon which selection acts – a fundamental premise of the field. In the field of human health and DOHaD, accurate estimates of association have direct implications for intervention strategies and health policy.

CHAPTER 3

EARLY LIFE SOCIAL AND ECOLOGICAL DETERMINANTS OF GLOBAL DNA METHYLATION IN WILD SPOTTED HYENAS

Zachary M. Laubach, Christopher D. Faulk, Dana C. Dolinoy, Luke Montrose, Tamara R. Jones, Donna Ray, Malit O. Pioon, and Kay E. Holekamp. 2019. Early life social and ecological determinants of global DNA methylation in wild spotted hyenas. *Molecular Ecology.* DOI: 10.1111/mec.15174

INTRODUCTION

The Developmental Origins of Health and Disease (DOHaD) hypothesis suggests that environmental conditions over the course of ontogeny have lasting effects on an organism's phenotype (Gillman, 2005). Of particular interest in DOHaD are vulnerable developmental stages ("sensitive periods") marked by high phenotypic plasticity (Heindel & Vandenberg, 2015) such as the periconceptional period, gestation, and the early post-natal period (Gluckman, Cutfield, et al., 2005; Hanson & Gluckman, 2014). The central premise of DOHaD is that environmental factors, two of the most widely studied being social experiences (Loi, Del Savio, & Stupka, 2013) and nutrition (Laubach et al., 2017), during sensitive periods of development have a larger impact on phenotypes than those occurring during other life stages (Bateson et al., 2004; Ben-Shlomo & Kuh, 2003).

DNA methylation as a pathway of DOHaD phenomena

One molecular pathway hypothesized to underlie DOHaD phenomena involves DNA methylation (Waterland & Michels, 2007). Among mammals, DNA methylation primarily refers to a methyl group that is covalently bonded to the fifth carbon of a cytosine base found primarily in cytosine-phosphate-guanine (CpG) dinucleotide pairs (Razin & Riggs, 1980). Of particular relevance to DOHaD is the fact that DNA methylation is a well-characterized and mitotically stable epigenetic modification that is both responsive to environmental factors, and associated with gene regulation and phenotype (Klose & Bird, 2006; E. Li & Bird, 2007). When considering the potential biological impact of DNA methylation, a key consideration is that its biological function (e.g. permissive vs. repressive effects on gene expression) depend in large part on where DNA methylation occurs within the genome – i.e., in repetitive elements, gene promoter regions, or gene bodies (Jones, 2012; Schübeler, 2015).

Of particular interest in this paper is DNA methylation of CpG sites in CCGG motifs located throughout the mammalian genome. In a cross-species comparison, we identified 2.19 million CCGG motifs in the dog canFam3 genome assembly (Lindblad-Toh et al., 2005), 2.75 million CCGG motifs in the cat felCat8 genome assembly (Lindblad-Toh et al., 2011), and 2.46 million CCGG motifs in the human hg38 genome assembly (Lander et al., 2001). Approximately 97% of CCGG motifs in the human genome occur in gene bodies and repetitive sequence regions of DNA, away from transcription start sites (Ball et al., 2009; Kinney et al., 2011). Taken together, there appears to be broad conservation of the CCGG motif across mammalian taxa and this motif is apparently distributed throughout the genome. At this scale, we and others (Vryer & Saffery, 2017) refer to this metric as global DNA methylation, as it is a composite

average of methylation sampled from CpG sites ubiquitously dispersed across the genome. Global DNA methylation is distinct from "genome-wide DNA methylation," which refers to DNA methylation measured across the genome at region-specific or single-nucleotide resolution. In general, global DNA methylation is thought to be an indicator of genomic stability (Schulz, 2006; Slotkin & Martienssen, 2007), as genome-wide hypomethylation is associated with high mutation rates and human cancers (R. Z. Chen, Pettersson, Beard, Jackson-Grusby, & Jaenisch, 1998; Feinberg & Vogelstein, 1983; Woo & Kim, 2012).

Beyond its relevance to health outcomes, global DNA methylation has also garnered interest as a biomarker of environmental exposures, thereby serving as a potential pathway linking experiences to phenotype. In humans, the nutritional environment and socioeconomic circumstance during early life, namely gestation (Boeke et al., 2012) and early childhood (Perng et al., 2012), are associated with global DNA methylation measured at LINE-1 repetitive elements. In rodents, maternal treatment with a synthetic stress hormone (betamethasone) causes a decrease in offspring global DNA methylation (Crudo et al., 2012), suggesting that social stressors that increase natural glucocorticoid level might also affect global DNA methylation. Rodent models also provide evidence that maternal nutritional supplementation is associated with global DNA methylation measured in offspring fetal tissues (Kovacheva et al., 2007; Kulkarni et al., 2011).

Despite the numerous human and rodent studies assessing DNA methylation within the context of the DOHaD hypothesis, there is a need to combine approaches used in biomedical research with research done in wild animals (Lea et al., 2017). Integration of DOHaD concepts (e.g., life course biology) with molecular data (e.g., DNA methylation) is especially salient in

long-lived gregarious species. Such efforts in wild animals could better enable investigators to explore not only how naturally-occurring environmental factors might affect DNA methylation, but also, the extent to which variation in DNA methylation patterns is detectable across the life span (Laubach et al., 2018). These efforts will ultimately pave the road for studies evaluating the relationships among environmental factors, DNA methylation, phenotype, and fitness, which are relevant in an evolutionary context given that variations in phenotype and health are shaped by natural selection (Laubach et al., 2018).

Objectives and hypotheses

In the present study, we test the hypothesis that early life social and ecological factors are determinants of global DNA methylation (%CCGG methylation) in three key age classes (cub, subadult, and adult) in a population of wild, spotted hyenas (*Crocuta crocuta*). For the early life social/ecological factors, we focus primarily on the social rank of each individual hyena's mother during the year in which it was born as our primary explanatory variable of interest ("maternal rank"). This rationale stems from the fact that social rank is a known determinant of priority of access to resources (Frank, 1986; Holekamp, Smith, Strelioff, Van Horn, & Watts, 2012; Tilson & Hamilton, 1984) and fitness (Höner et al., 2010; Swanson, Dworkin, & Holekamp, 2011) in spotted hyenas. In addition, we also consider litter size, extent of anthropogenic disturbance during the hyena's birth year, and prey availability. We predicted positive associations of both maternal rank and prey density during early life with global DNA methylation. We also predicted that larger litter size and more exposure to human disturbance during the hyena's birth year would be associated with lower global DNA methylation. For all

relationships of interest, we anticipated larger magnitude of associations during earlier than later age classes given that explanatory variables were measured during a hyena's birth year.

METHODS

Study population

We used samples and data collected by personnel from the Mara Hyena Project, a long-term field study of wild spotted hyenas in the Masai Mara National Reserve, Kenya. Spotted hyenas are gregarious carnivores that live in large groups known as clans (Kruuk, 1972). Within each clan, relationships among individuals are structured by a linear dominance hierarchy organized by matrilines, and a cercopithecine primate-like pattern of youngest ascendency during the process of rank acquisition (Engh, Esch, Smale, & Holekamp, 2000; Holekamp & Smale, 1991; Holekamp & Smale, 1993; Smale, Frank, & Holekamp, 1993). A hyena's rank determines not only its priority of access to such critical resources as food and mates, but also the nature of its social interactions with other clan members; both resource access and social interaction patterns are known to affect fitness in this species (Frank, 1986; Holekamp et al., 2012; Holekamp, Smale, & Szykman, 1996; Smith, Memenis, & Holekamp, 2007). Female hyenas typically give birth to 1-2 offspring (Frank, Glickman, & Licht, 1991; Holekamp et al., 1996), which depend on their mothers for food and protection until offspring are approximately two years of age (Watts et al., 2009). The importance of social status in hyena societies, and the protracted period of maternal dependence, make this species a good model system in which to test our hypothesis.

Demographic, behavioral, and biological sample data have been collected continuously since 1988 from individual hyenas identifiable by their unique spot patterns. For the present analysis, we selected a subset of 381 hyenas for which we have both detailed behavioral data for calculation of maternal rank (the primary explanatory variable of interest) and archived blood samples for quantification of global DNA methylation (the dependent variable of interest). After completing Quality Assessment and Quality Control (QA/QC) of DNA methylation values, our final analytic sample comprised 293 individual hyenas belonging to six clans (see Supplemental Material). Of these individuals, 58 had repeated measures capturing more than one age class due to the opportunistic nature of immobilizations and blood draws.

Explanatory variables: early life social environment, ecological factors, and life history traits

Early life social environment

Maternal rank

We determined the social rank of each adult female based on her wins and losses in dyadic agonistic interactions (Engh et al., 2000; Holekamp & Smale, 1993; Smale et al., 1993). Each individual's rank was updated annually. To characterize the early life social environment, each cub was assigned the rank held by its mother, called its maternal rank, during the year in which it was born. In order to account for differences in clan size and yearly demographic changes, we standardize rank on a relative scale from -1, corresponding to the lowest ranking adult female, to 1, corresponding to the highest-ranking female.

Litter size

In addition to interacting with its mother, each young hyena also interacts and competes with its littermate, if it has one (Frank et al., 1991; Holekamp et al., 1996). Based on daily observations of our study clans, we determined whether each hyena belonged to singleton or twin litter when it was first seen above ground.

Ecological factors

Anthropogenic disturbance during the birth year

We categorized anthropogenic disturbance based on the amount of illegal livestock grazing in the Reserve by pastoralist Masai herdsman under two different management regimes. Based on data collected and analyzed by Green et al. (2018), we assigned hyenas in each clan in each year to one of three categories of human disturbance: high, medium, and low (Green et al., 2018). Levels of human disturbance were based on livestock counts that began in 2000 in the eastern part of the reserve near the Masai town of Talek. The counts were conducted systematically throughout the year and the total number of livestock counted were averaged annually. Livestock were never observed on the western side of the reserve, and illegal grazing did not proliferate near Talek until around 2000 (personal observation).

Prey density during discrete developmental periods

Twice each month, research assistants counted all prey animals observed within 100 meters of either side of established 4-km prey transect routes in the territories of our study clans. Details of these methods are presented elsewhere (Cooper, Holekamp, & Smale, 1999;

Green et al., 2018). We combined counts of impala (*Aepyceros melampus*), plains zebra (*Equus burchelli*), Thomson's gazelle (*Eudorcas thomsonii*), topi (*Damaliscus lunatus*), and white-bearded wildebeest (*Connochaetes taurinus*), which are the primary wild ungulate prey of hyenas in the Reserve, comprising at least 93% of the prey hunted by hyenas there (Holekamp, Smale, Berg, & Cooper, 1997). We estimated the average prey density during five discrete 3-month periods in the hyena's early life so that we could identify sensitive periods for exposure to varying nutritional regimes. For each hyena from our study population, we calculated the average number of ungulate prey during the peri-conceptional period (1.5 months before and 1.5 months after conception), during gestation (3 months prior to birth), from birth to 3 months, 3-6 months, and 6-9 months. These five periods were selected because they cover key developmental periods, starting with their mother's access to food before conception, covering the 110-day gestation period, and extending through early post-natal ontogeny (Holekamp & Smale, 1998; Kruuk, 1972). Our approach to modeling associations of food availability at discrete time periods during gestation and early life with later life phenotypes was intended to parallel an analytical approach used by researchers studying the Dutch Hunger Famine (Painter et al., 2005).

Life history traits

Sex

We determined the sex of each hyena based on the glans morphology of its erect phallus during field observations; this is reliable starting at 3 months of age (Frank, Glickman, & Powch, 1990).

Age

We aged hyenas by back calculating their birthdates based on their physical appearance when first observed as infants. Based on their pelage, morphology and behavior, we are able to determine a cub's age with an accuracy of ± 7 days (Holekamp et al., 1996). We used this method to determine each hyena's age in months at the time of blood collection. Because we were interested in associations of birthyear socioecological factors on DNA methylation among different age classes of hyenas, we also operationalized age at blood collection as a 3-level variable – cub, subadult, and adult – corresponding to prominent life-history milestones during development. We defined the cub age class as ≤12 months of age (Holekamp & Smale, 1998), which approximately coincides with the mean age of weaning (11.9 months) in this subsample of our study animals. The subadult age class was defined as >12 to ≤24 months of age. The adult age class was classified as >24 months of age, as hyenas become reproductively competent at 24 months (Holekamp & Smale, 1998; Holekamp et al., 1996).

Dependent variable: global DNA methylation

Blood collection and DNA extraction

Hyenas were immobilized using 6.5 mg/kg of tiletamine-zolazepam (Telazol ®) delivered in a pressurized dart fired from a CO_2 powered rifle (Telinject Inc.). We collected blood from the hyena's jugular vein into ethylenediaminetetraacetic acid (EDTA) coated vacuum tubes. The samples were flash frozen in liquid nitrogen or processed for genomic DNA extraction (using the Gentra Pure Gene kit by Qiagen®) then stored in -80°C freezers until time of analyses.

At the time that we selected blood samples for DNA methylation assays, we noted the date of sample collection and calculated sample age, which was included in a sensitivity analysis to assess whether or not there was potential variation in DNA quality due to storage time.

Global DNA methylation assay

We quantified global DNA methylation as percent methylated CCGG sites (%CCGG methylation) in peripheral leukocytes using LUMA (Karimi, Johansson, & Ekström, 2006; Karimi et al., 2006). Extensive details on our laboratory methods and QA/QC are included in the Supplemental Material. Briefly, this method uses both methyl sensitive (*HpaII*) and methyl insensitive (*MspI*) restriction enzymes that target a shared recognition motif of CCGG throughout the genome. In mammals, there are roughly 2.4 million CpG sites at CCGG motifs. Generalizing among mammals by using the well-annotated human genome, approximately 3% of CpG sites belonging to the CCGG motif are near (< 1kb) transcription start sites, 45% are in gene bodies, and 52% are in non-coding repetitive elements (Ball et al., 2009; Kinney et al., 2011). Given the high proportion of CpG sites within gene bodies and non-coding repetitive elements, we suspect that higher %CCGG methylation measured via the LUMA assay may reflect regulation of transcription and alternative splicing (Lev Maor, Yearim, & Ast, 2015; Li, Zhang, Huang, & He, 2018) as well as repression of repetitive elements (Barau et al., 2016; Coluccio et al., 2018) and enhanced chromosomal stability (Eden, Gaudet, Waghmare, & Jaenisch, 2003; Tuck-Muller et al., 2000). Accordingly, we cautiously interpret higher %CCGG methylation as a more favorable outcome than lower %CCGG methylation.

Statistical analyses

Prior to formal analysis, we performed a series of quality control assessments and evaluation on our data. First, we examined the distribution of continuous variables (%CCGG methylation, prey density, age in months), and assessed frequency of nominal categorical variables (sex, maternal rank quartiles, litter size [singleton vs. twin], human disturbance during birth year [low, medium, high]) for deviations from normality, and to identify missing values. Next, given the potential impact of shared genes among siblings on DNA methylation (Hannon et al., 2018), we calculated intraclass correlations (ICC) comparing within and between family variability in %CCGG methylation based on the premise that an ICC >0.1 indicates greater within than between family correlation (i.e., lower within than between family variability) which would warrant a need to account for shared genes in the analysis. Third, because sex (Doherty et al., 2016) and age (T. H. Bjornsson et al., 2008) can potentially alter the relationship between early exposures and DNA methylation, we assessed for effect modification by sex and by age on the relationship between maternal rank (our primary explanatory variable of interest) and %CCGG methylation using linear mixed models. We accounted for the repeated measurements of DNA methylation from the 58 individuals with more than one DNA methylation value by including a random intercept for hyena ID. Here, we considered stratified analysis if the P-value for the interaction term was <0.20. The tests for interaction indicated effect modification of the relationship between maternal rank and offspring %CCGG methylation by age group, so we carried out subsequent analyses separately for cub, subadult, and adult hyenas. Finally, we examined bivariate associations between the explanatory variables and %CCGG methylation among all hyenas in the study. We conducted bivariate analysis using a linear mixed model with

a random effect for individual identity (to account for repeated measurements) to explore crude associations between our explanatory variables and %CCGG.

For the main analysis, we examined associations between each explanatory variable and %CCGG methylation separately for cubs (n = 65), subadults (n = 127), and adults (n = 127). We employed this analytical strategy to explore the extent to which early life environment was associated with DNA methylation at different stages of development. We acknowledge that we have three cross-sectional populations rather than one longitudinal population due to constraints on available archived samples. In the analysis, we used linear regression models to examine unadjusted and adjusted associations between each explanatory variable and %CCGG methylation within each life-stage category. In the adjusted models, we explored the extent to which each of the explanatory variables was associated with %CCGG methylation after controlling for key covariates, including a hyena's continuous age in months at the time of darting and sex (Model 1). We assessed residual plots for each multiple variable regression model and conducted a Breusch-Pagan test to check for violations of homoskedasticity.

In adults only, we ran an additional model in which maternal rank was the explanatory variable of interest and %CCGG methylation was the continuous outcome. This model, which was limited to adult females, included continuous age in months as a covariate and each hyena's own rank during the year in which it was darted. Inclusion of the hyena's own rank allowed us to assess the independent effects of maternal rank after hyenas had taken their places in the rank hierarchy.

In models where prey density was the explanatory variable of interest, we also controlled for prey density during all previous developmental periods to isolate the

independent effect of the period of interest. That is, we treated earlier prey density as a confounding variable that, if not controlled for in our model, could bias our estimate of association for current prey density and offspring DNA methylation.

Finally, based on results of our adjusted Model 1 for each age group, we implemented Model 2, which mutually adjusted for statistically significant (P<0.05) explanatory variables from Model 1. That is, Model 2 included all of the covariates (e.g. sex and age in months) in addition to all explanatory variables that were significantly associated in Model 1 with %CCGG methylation. By doing this, Model 2 enabled us to document the independent effects of the strongest determinants of %CCGG methylation.

Sensitivity analyses

In sensitivity analyses, we evaluated the potential impact of sample storage time on DNA methylation measurement by additionally including the year during which DNA was extracted and put into our freezer as a covariate in the models. In addition to comparing the direction, magnitude, and precision of the estimates, we also calculated the variance inflation factor (VIF) to test for collinearity among covariates given that both sample age and anthropogenic disturbance are both based on the time order of years during the project.

RESULTS

Descriptive statistics

Slightly more than half the study population were females (56%), and we had more samples from individuals at older than young life-stages; 20% were cubs, 40% were subadults,

and 40 % were adults. Most sampled individuals (79%) were members of twin litters and 21% were singletons. Additional sample characteristics are shown in Table 3.1. Our indicator of global DNA methylation, %CCGG methylation in peripheral leukocytes, was relatively normally distributed with a mean ± SD of 75.75 ± 2.79% (Figure 3.1). In bivariate analysis, there was no significant difference in %CCGG methylation between male and female hyenas (males 75.57 ± 3.09 and females 75.89 ± 2.55 %CCGG methylation; difference = -0.30 [95% CI: -0.93, 0.33], P-value = 0.35). We noted a positive monotonic relationship between hyena age category and %CCGG methylation: 74.90 ± 3.64% in cubs, 75.82 ± 2.60% in subadults, and 76.12 ± 2.38% in adults (F-statistic = 4.58, P-value = 0.02).

	N †	%
LIFE HISTORY TRAITS		
Sex		
Female	163	56%
Male	129	44%
Life stage		
Cubs (mean age = 10.0 ± 1.5 months)	65	20%
Subadults (mean age = 16.9 ± 3.2 months)	127	40%
Adults (mean age = 60.6 ± 32.9 months)	127	40%
EARLY LIFE SOCIAL ENVIRONMENT		
Maternal rank during birth year		
Q1 (Lowest)	65	26%
Q2	63	25%
Q3	59	24%
Q4 (Highest)	62	25%
Litter size		
Singleton	44	21%
Twins	164	79%
ECOLOGICAL CHARACTERISTICS		
Anthropogenic disturbance during birth year‡		
Low	102	36%
Medium	96	34%
High	87	30%
Average prey density during discrete developmental periods (per 1 km^2)§	**N †**	**Mean ± SD**
Periconception	237	237.3 ± 162.6
Gestation	230	237.6 ± 188.6
Birth to <3 months of age	226	205.1 ± 133.4
3 to <6 months of age	215	222.2 ± 126.9
6 to <9 months of age	217	255.2 ± 200.0

† 320 measurements from 293 individual hyenas; Ns may not add up to 293 individuals, due to missing values.

‡ Human presence was determined by counts of livestock within the reserve boundary and proximity to Masai villages.

§ Prey species include the 5 most commonly consumed wild ungulates: Impala, Thomson's gazelle, Topi, Plains Zebra, and Wildebeest.

Table 3.1. Life history and social characteristics of 293 spotted hyenas as well as ecological measures from the Masai Mara, Kenya.

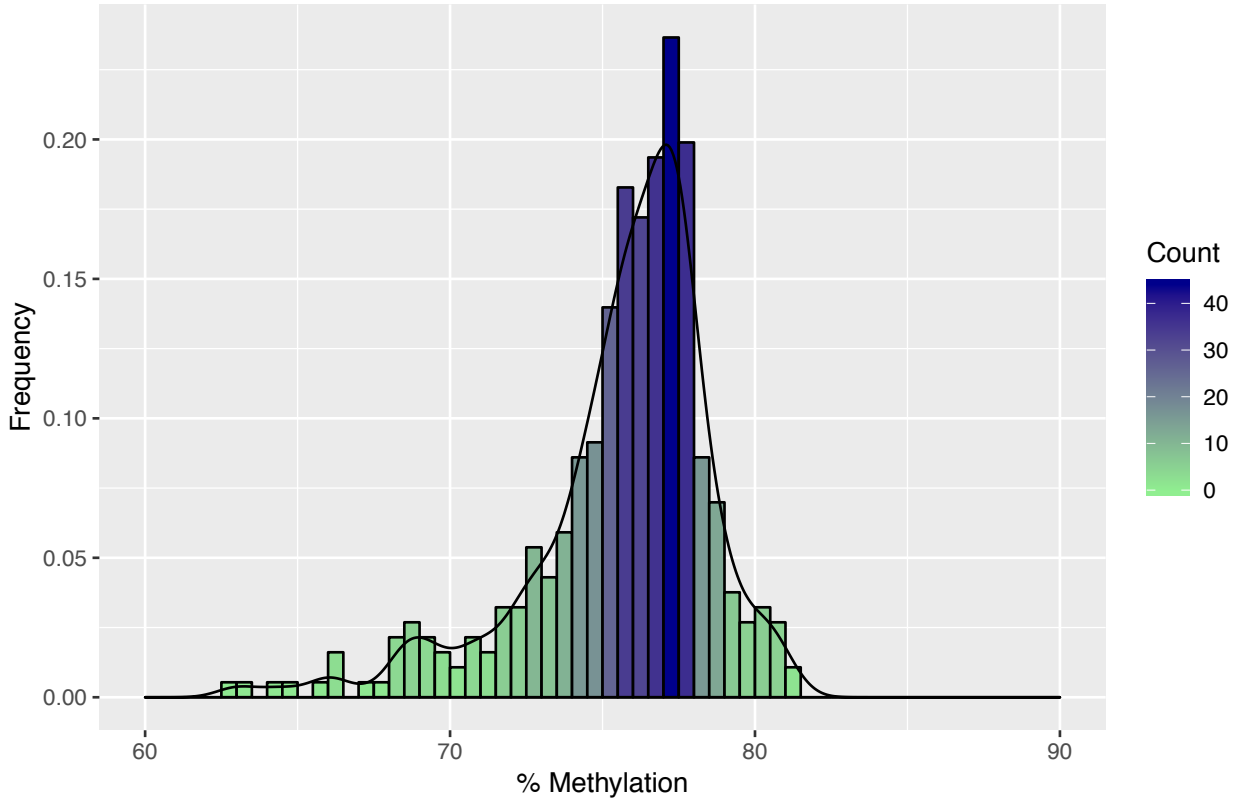

Figure 3.1. Frequency distribution of %CCGG methylation, our continuous outcome of interest, for hyena genomic DNA samples that were extracted from whole blood from 293 individual hyenas and assayed with LUMA.

Data checks

We found no evidence of familial clustering, with ICCs of 0.052, 0.077, and 0.000 in cubs, subadults, and adults, respectively. Given that an ICC >0.1 is considered the cut-off for the need to cluster by a variable (Vajargah & Masoomehnikbakht, 2015), these low ICCs suggest that familial clustering is not an issue in our data, and thus, not accounted for in the models.

We also tested for a statistical interaction between sex and age class (cub, subadult, and adult) with maternal rank (our primary explanatory variable of interest) on %CCGG methylation, which revealed evidence of effect modification with age (*P*-interaction = 0.06) but not sex (*P*-interaction = 0.42). Given the effect modification with age, in addition to our *a priori* interest in

investigating the extent to which associations between early experiences and DNA methylation are observed across development, we stratified all subsequent analyses by age class. Because the relationship between maternal rank and %CCGG methylation in cubs was not monotonic (Figure 3.2), we binned standardized maternal rank into quartiles, with the first quartile representing lowest maternal rank and the fourth quartile representing highest maternal rank.

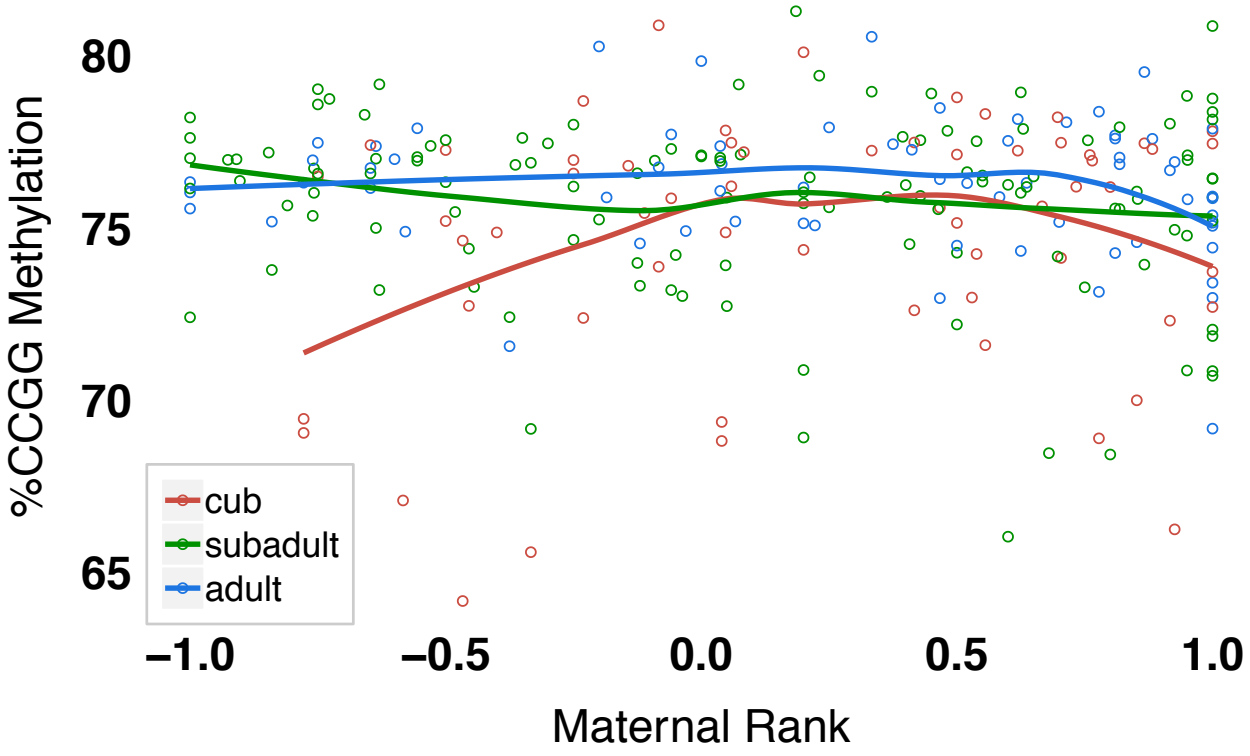

Figure 3.2. Hyena %CCGG methylation by standardized maternal rank (-1 lowest rank, and 1 highest rank) and stratified by age categories of cubs, subadults, and adults.

Prior to our age stratified analyses, we also ran a model in which %CCGG methylation is the dependent variable, and explanatory variables included: offspring sex, maternal rank, offspring age groups (cub, subadult, and adult) and a maternal rank*offspring age group interaction term. While the beta estimates from the interaction model are more limited in their

interpretation than the stratified models discussed below, there was concordance between these results (Supplemental Table 3.1).

Cub models

Table 3.2 shows the Model 1 adjusted associations between explanatory variables and %CCGG methylation in hyenas during the cub life-stage (for unadjusted estimates, see Supplemental Table 3.2).

	β (95% CI) for %CCGG methylation					
	Cub models[†]	P	Subadult models[†]	P	Adult models[†]	P
EARLY LIFE SOCIAL ENVIRONMENT						
Maternal rank during birth year						
Q1 (Lowest)	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Q2	**3.19 (0.68, 5.71)**	**0.016**	-0.50 (-1.78, 0.78)	0.444	0.52 (-0.85, 1.88)	0.461
Q3	**3.46 (0.96, 5.97)**	**0.009**	-0.47 (-1.78, 0.85)	0.486	0.81 (-0.59, 2.21)	0.261
Q4 (Highest)	1.68 (-0.96, 4.32)	0.217	-0.72 (-2.06, 0.62)	0.296	-0.64 (-1.94, 0.65)	0.333
Litter Size						
Singleton	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Twins	0.78 (-1.42, 2.98)	0.490	*-1.26 (-2.69, 0.17)*	*0.088*	-0.08 (-1.51, 1.35)	0.911
ECOLOGICAL FACTORS						
Anthropogenic disturbance during birth year						
Low	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Medium	**2.88 (0.99, 4.78)**	**0.004**	0.84 (-0.44, 2.13)	0.201	0.54 (-0.56, 1.63)	0.340
High	**3.51 (0.83, 6.19)**	**0.013**	**2.05 (0.85, 3.25)**	**0.001**	0.80 (-0.49, 2.09)	0.229
Prey density during discrete developmental periods (per 1 SD)[‡, §]						
Periconception	**-1.18 (-2.03, -0.33)**	**0.009**	0.03 (-0.53, 0.60)	0.906	-0.02 (-0.51, 0.46)	0.928
Gestation	-0.41 (-1.17, 0.34)	0.287	0.30 (-0.59, 1.18)	0.513	-0.05 (-0.55, 0.46)	0.861
Birth to <3 months of age	**-1.40 (-2.44, -0.36)**	**0.011**	-0.37 (-0.93, 0.19)	0.197	**-0.68 (-1.14, -0.22)**	**0.005**
3 to <6 months of age	0.55 (-0.51, 1.61)	0.315	0.32 (-0.19, 0.83)	0.220	0.15 (-0.42, 0.72)	0.608
6 to <9 months of age	0.01 (-0.96, 0.97)	0.991	0.01 (-0.70, 0.72)	0.978	-0.06 (-0.55, 0.43)	0.806

[†] Models are adjusted for hyena age at blood collection (months) and sex.

[‡] Prey species include the 5 most commonly consumed wild ungulates: Impala, Thomson's gazelle, Topi, Plains zebra, and Wildebeest.

[§] Models are adjusted for prey densities from previous developmental periods.

Bold estimates are significant at p<0.05, and italicized estimates are significant at p<0.1

Table 3.2. Model 1 adjusted associations of explanatory variables with global DNA methylation in hyenas assessed at each age category.

In Model 1, which accounted for the hyena's age (in months) and sex, maternal rank was positively associated with %CCGG methylation. Specifically, hyena cubs whose mothers were in the second, third, and fourth quartiles of rank had 3.19 (95%CI: 0.68, 5.71; P = 0.016), 3.46 (95%CI: 0.96, 5.97; P = 0.009), and 1.68 (95%CI: -0.96, 4.32; P = 0.217) higher % CCGG

methylation, respectively, than those whose mothers were in the lowest rank quartile. The relationship between maternal rank and %CCGG methylation was positive but not strictly monotonic. We also found that, compared to cubs born into low anthropogenic disturbance, cubs from medium anthropogenic disturbance groups had 2.88 (95%CI: 0.99, 4.78; P = 0.004) %CCGG higher methylation and cubs born into high disturbance had 3.51 (95%CI: 0.83, 6.19; P = 0.013) %CCGG higher methylation. On the other hand, density of wild ungulate prey periconceptionally and from birth to 3 months of age was inversely related to %CCGG methylation. In unadjusted analysis, we found that for every 1 SD of wild ungulate prey density measured periconceptionally there was 1.24 (95%CI: 0.43, 2.04; P = 0.004) lower %CCGG methylation, and each 1 SD of wild ungulate prey density measured from birth to 3 months corresponded to 1.55 (95% CI: 0.53, 2.58; P = 0.004) lower %CCGG methylation. Adjusting for hyena's age, sex, and previous prey period densities in Model 1 slightly attenuated the associations at periconception (-1.18 [95% CI: -2.03, -0.33]; P = 0.009), and from birth to 3 months (-1.40 [95% CI: -2.44, -0.36]; P = 0.011). In Model 2, we mutually adjusted each significant explanatory variable from our previous models (Model 1) by including maternal rank, anthropogenic disturbance, and average wild ungulate prey density from periconceptional, gestational, and birth to 3 months as fixed effects parameters in the same model. Doing so attenuated estimates for anthropogenic disturbance and prey density from periconception to 3 months but not for maternal rank (and in fact, slightly strengthened the associations involving maternal rank), nor did it substantially widen the confidence intervals for maternal rank or wild ungulate prey density (Figure 3.3).

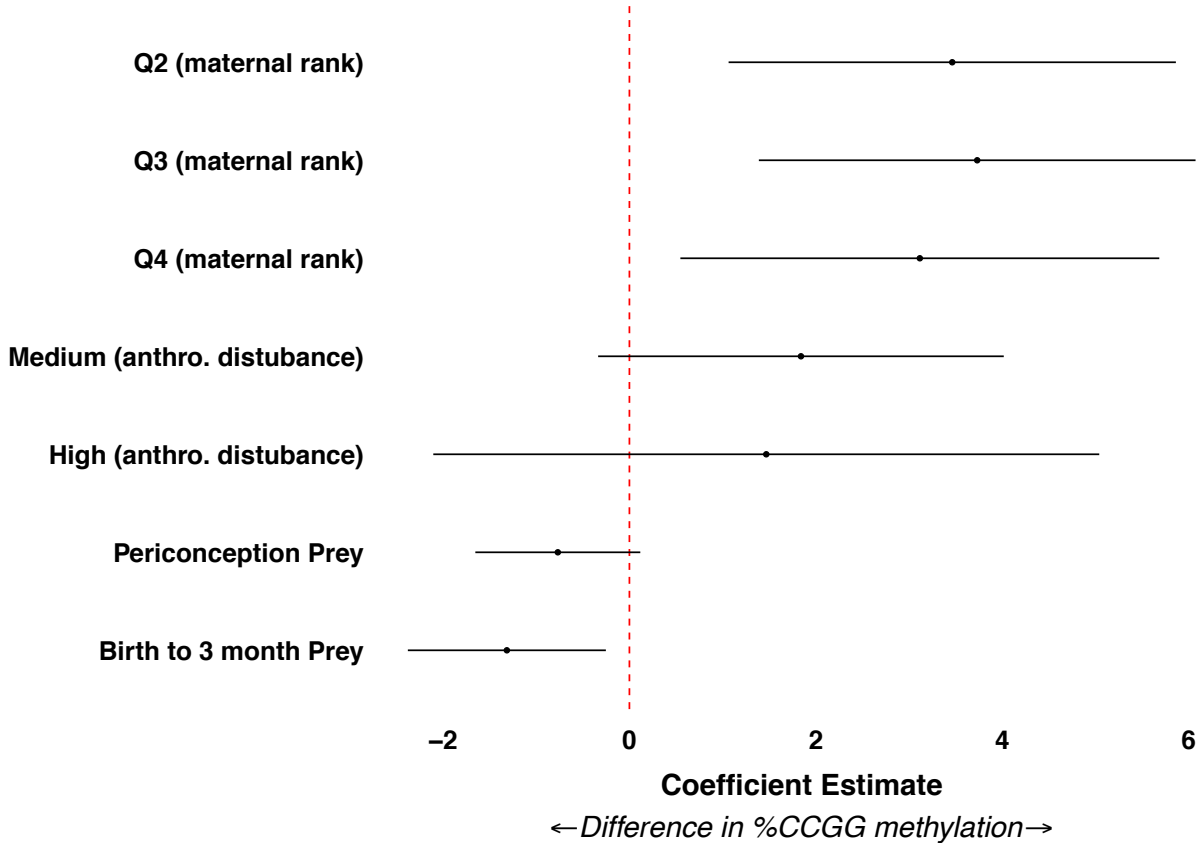

Figure 3.3. Beta estimates and 95% CI for %CCGG methylation from Model 2 in cubs (n = 57). Model 2 included all significant explanatory variables from Model 1 including: maternal rank, anthropogenic disturbance, wild ungulate prey density at periconception and from birth to 3 months, as well offspring age at the time of darting and sex.

Subadult models

Table 3.2 also shows results for subadult hyenas. We did not observe any statistically significant associations between maternal rank and %CCGG methylation in this age group after adjusting for covariates in Model 1. As with our cub models, we found that medium and high anthropogenic disturbance corresponded to 0.84 (95% CI: -0.44, 2.13; P = 0.201) and 2.05 (95% CI: 0.85, 3.25; P = 0.001) higher %CCGG methylation, respectively. We also noted a trend

toward (1.26; 95% CI: -0.17, 2.69; P = 0.088) lower % CCGG methylation in twin than singleton litters.

Adult models

In the last column of Table 3.2, we show associations for adult hyenas. In Model 1, we again observed no effect of maternal rank on %CCGG methylation. In a subset of adult females, we ran an additional model that controlled for each hyena's own rank and similarly found no significant effect of maternal rank on %CCGG methylation (Supplemental Table 3.3). However, there was an inverse association between wild ungulate prey density from birth to 3 months of age and %CCGG methylation. Each 1 SD increment in prey density corresponded with 0.68 (95% CI: 0.22, 1.14; P = 0.005) lower % CCGG methylation. None of the other early life social or ecological variables were related to global DNA methylation in this age group.

Sensitivity analyses

Results from models where we further adjusted for sample age as a covariate were similar to those without sample age adjustment (Supplemental Table 3.4). These findings, in conjunction with potential collinearity between sample age and some of the explanatory variables of interest (particularly in models for anthropogenic disturbance which had VIFs > 5.0) and a recent publication demonstrating the robustness of DNA methylation to storage time (Y. Li et al., 2018), serve as the impetus for us to focus the discussion of results on models that do not include sample age.

DISCUSSION

In this study of 293 wild cub, subadult and adult spotted hyenas in Kenya, we sought to identify early life social and ecological explanatory variables of global DNA methylation, as indicated by % methylated CCGG across the genome. In line with our expectations, we found that higher maternal rank at birth was associated with higher global DNA methylation in offspring sampled as cubs, but not in those sampled as subadults or adults. Among cubs and subadults, higher anthropogenic disturbance during the year in which hyenas were born corresponded to greater methylation. We also found an unexpected inverse relationship between prey density (an indicator of food availability) measured during the peri-conceptional period through the first three months of life and global DNA methylation in offspring sampled as cubs and adults. Associations in cubs were robust to mutual adjustment, suggesting independent effects of perinatal social environment and food availability on later life global DNA methylation.

Comparison of %CCGG methylation in hyenas to that in other mammals

A comparison of %CCGG methylation of DNA extracted from whole blood using the LUMA assay shows that hyenas have similar global methylation to other vertebrates, including both another member of the order Carnivora and humans. For example we observed only 0.7% less methylation in hyenas than domestic dogs (Montrose et al., 2015), and hyenas had approximately 3.7% greater methylation than humans (Ono et al., 2012; Virani et al., 2012).

Maternal rank and global DNA methylation

Our most notable finding was a positive, albeit not strictly monotonic, relationship between maternal rank and global DNA methylation in cubs. Specifically, we found that cubs born to mothers in the upper three rank quartiles had 2-3% higher CCGG methylation than those whose mothers were in the lowest rank quartile. This association may reflect the fact that offspring of high-ranking mothers have greater access to social capital and resources, which in other gregarious species, predict positive health outcomes (Sapolsky, 2005). Work on rhesus macaques (*Macaca mulatta*) revealed differences in DNA methylation at more than 25,000 genomic locations in placental tissue when comparing offspring from high- and mid-rank mothers to those of low-ranking mothers (Massart et al., 2017). Similarly, differential DNA methylation across the genome was also observed in a recent human study that reported associations between socioeconomic status (SES) and DNA methylation at nearly 500 CpG sites in young children (Bush et al., 2018b). Using a more focused candidate gene approach, three studies of humans quantified methylation of genes involved in growth (King, Murphy, & Hoyo, 2015; Obermann-Borst et al., 2012) and regulation of stress hormones (Appleton et al., 2013) in cord and infant blood, and found variation in gene-specific DNA methylation with maternal education and household income, which are both strong indicators of SES. Taken together, these studies point toward an effect of early life social status on DNA methylation patterns that is detectable as early as the day of birth. Of particular relevance to the present study are findings from school-age children that higher family SES level was associated with higher global DNA methylation (LINE-1 repetitive element) in boys (Perng et al., 2012). These findings are pertinent to our results given not only similarities in the types of independent (social status)

and dependent variables (global DNA methylation) of interest, but also considering that both studies assessed social status early in life and metrics of global DNA methylation in post-natal juveniles.

We did not observe any relationship between maternal rank and %CCGG methylation among subadult or adult hyenas, even after controlling for adult hyenas' own ranks. There are a few potential explanations for the null findings in later life-stages. First, the epigenome is labile and responsive to the environment across ontogeny. In this particular study, %CCGG methylation, presumably established in association with maternal rank at birth, may be further modified in response to a hyena's own rank and related social or ecological factors, especially during later life-stages when the hyena becomes less dependent on its mother. Studies in rhesus macaques (Tung et al., 2012a) and humans (Mcguinness et al., 2012) have reported marked variation in genome-wide and global DNA methylation in adulthood with respect to current social rank and SES, respectively, suggesting potential effects of one's current social environment on the epigenome. Similarly, Subramanyam et al. found no relationship between early life SES and adult global DNA methylation (LINE-1 and ALU repetitive elements) in 998 participants of a large multi-ethnic population of middle-aged adults in the U.S. However, the authors did find that attained wealth, a socioeconomic asset accrued across the life span, was associated with higher methylation of both LINE-1 and ALU (Subramanyam et al., 2013). A subsequent study of the same subject population investigated effects of early life and adult SES on gene-specific methylation, and found that SES at both time-points was associated with differential methylation – with both positive and negative directions of associations – of specific genes in adulthood, although the subset of genes affected by childhood and adult SES did not

completely overlap (Needham et al., 2015). Together these findings suggest that, although social status clearly affects the epigenome, these effects likely vary not only across different life stages, but also, with respect to detectable differences in DNA methylation assessed at specific loci vs. at the global level. Further, effects of SES that are apparent early in life may not persist throughout ontogeny. In the present study, our *a priori* hypothesis focused on the effect of the early life environment on later DNA methylation. However, we did consider the potential effects of a hyena's own rank given that this might contribute to the null associations that we observed during later life-stages. Nevertheless, controlling for an adult hyena's own rank did not reveal a significant effect of maternal rank on %CCGG methylation in adults.

Another potential explanation for the null findings in subadults and adults revolves around the fact that recapitulation of DNA methylation patterns is not perfect. That is, DNA methylation may change over time due to random errors. A longer time elapsed from original establishment of DNA methylation patterns *in utero* corresponds to greater potential for errors to occur in DNA methylation replication mechanisms (Laubach et al., 2018).

Anthropogenic disturbance

Mid- and high-level human disturbance, based on the year in which a hyena was born, were positively associated with cub and subadult global DNA methylation. Although we expected that human disturbance would be negatively associated with global DNA methylation, we observed a positive anthropogenic disturbance effect that appeared to be strongest among cubs and was evident in subadults. Regardless, these findings are interesting from a biological viewpoint given that hyenas are generalist hunters that thrive under medium disturbance

(Cooper et al., 1999; Green et al., 2018). It may be that higher levels of anthropogenic activity enhance availability of livestock as prey for local hyenas. We know that Masai livestock are utilized as a food source by our study animals when they are available as potential prey (Green et al., 2018; Kolowski & Holekamp, 2006),and this nutritional abundance may be reflected in the epigenome.

Prey density

We observed an inverse association between prey density in the first three months of life and global DNA methylation in cubs and adults. This is the opposite of what we had hypothesized, given that dietary intake of methyl-donor nutrients provides the primary substrate for the DNA methylation reaction (O. S. Anderson, Sant, & Dolinoy, 2012). Although these results were unexpected, one potential explanation may involve increased social stress during periods of food abundance. In our study population, we have repeatedly noted an increase in the rate at which hyenas engage in social interactions (both positive or negative) during periods of greater prey abundance (eg., Holekamp et al., 2012). Furthermore, we found that fecal glucocorticoid levels are elevated during periods of higher prey abundance among juvenile but not pregnant adult female hyenas (Greenberg, 2017). Although greater prey abundance was not associated with higher stress levels in pregnant females in this analysis, it is possible that we were underpowered to detect an effect among pregnant females given that we had measurements from only 31 of them compared to 123 juvenile hyenas. Given this caveat, elevated stress hormones are known to be associated with DNA methylation. For example, an experimental study of guinea pigs revealed that *in utero* exposure to elevated

glucocorticoid levels caused lower global DNA methylation assessed via LUMA (Crudo et al., 2012). If a more powerful analysis reveals that pregnant female hyenas have elevated glucocorticoids during higher than lower prey abundance periods, then this could potentially explain the inverse association we observed between prey abundance and global DNA methylation. Second, in contrast to many other hyena populations in Africa, food is very seldom in short supply for Mara hyenas such that periods of low prey abundance experienced by this population do not induce nutritional stress in hyenas, at least not comparable to famine exposed humans (Heijmans et al., 2008b; Tobi et al., 2009).

Litter size

Besides the above-mentioned findings, there is another association worth noting. We observed that twins had lower global DNA methylation than singletons during the subadult life-stage, although this association was not statistically significant. That this effect is only observed in subadults makes sense in light of the fact that either social or nutritional stress from competition with a sibling may accumulate during the months prior to weaning and during the subadult life stage.

Strengths and limitations

Our study had a number of strengths, including its large sample size, the use of a novel, long-lived social mammal as a model organism, and the availability of rich meta-data on demographic, behavioral, and ecological factors that might influence DNA methylation. These

unique data coupled with biological samples collected from hyenas at different life stages allowed us to test DOHaD hypotheses in a wild animal system.

Our study also has clear limitations. First, we used the LUMA assay, which is a reliable and a particularly attractive option for wild animals lacking well curated genomes (Head, Mittal, & Basu, 2014). However, the CCGG sites targeted by this assay represent a single composite average of genomic DNA methylation and do not provide any information on finer resolution differences in DNA methylation that may be relevant to environmental risk factors and/or phenotypes. For example in humans, Waterland et al. (2010) found that individuals who were conceived during seasonal food shortages exhibited higher DNA methylation at metastable epialleles but no differences in global measures of DNA methylation (LINE-1) or DNA methylation of imprinted genes during childhood. Future studies using genome-wide approaches, such as Reduced Representation Bisulfite Sequencing (Meissner et al., 2005), are warranted to home in on specific regions of the genome that may demonstrate changes in DNA methylation related to the environment and/or phenotypes.

Another limitation is our use of archived DNA extracted from blood but without information on cellular heterogeneity (i.e., proportion neutrophils, eosinophils, basophils, lymphocytes, and monocytes), which may be relevant given that there is cell type-specific variation in DNA methylation (Adalsteinsson et al., 2012). However, we believe our measure of global DNA methylation, taken as an average across leukocyte cell types, is still valuable given that environmental exposures like social stress (Engler, Bailey, Engler, & Sheridan, 2004) and infection (Helmby, Jönsson, & Troye-Blomberg, 2000) affect the cellular composition and the

distribution of leukocyte subpopulations. Therefore, the “effects” of our explanatory variables on DNA methylation may well include their effects on cell type composition.

Other limitations include 1) the fact that our study design is cross-sectional (which does not allow assessment of within-individual change over time, and is generally prone to suffer from reverse causation and unmeasured confounding (Greally, 2018; Lappalainen & Greally, 2017)); 2) the potential for sample selection bias (e.g., offspring from low ranking lineages, which presumably have lower DNA methylation, are in worse condition and may be less likely to survive to older ages thus reducing variation in DNA methylation in the older age classes); and 3) a possible time-varying effect of our explanatory variables on the epigenome throughout a hyena’s life, thus limiting the extent to which we can identify causal relationships from data collected at specific time-points (Mansournia, Etminan, Danaei, Kaufman, & Collins, 2017). Finally, we cannot discount the possibility of chance findings given the number of models tested. However, our research focus was to describe and assess the direction, magnitude, and precision of the estimates rather than focus on statistical significance, especially in light of the fact that our explanatory variables were related biological concepts and included correlated variables, like prey density during successive time periods. In such scenarios, use of multiple comparisons corrections would unfairly penalize models containing correlated explanatory variables of interest and increase risk of type 2 error at the cost of reducing type 1 error (Rothman, 1990).

Conclusions

In conclusion, we found that maternal social rank at the time of birth was positively associated with %CCGG methylation in hyena cubs, but not in subadult or adult hyenas. We also found that higher anthropogenic disturbance at birth, which is possibly an indicator of a reliable and easy to catch food source (i.e. domestic livestock) corresponded with higher global DNA methylation in cubs and subadults. Finally, availability of wild ungulate prey at periconception (among cubs only) and from birth to 3-months of age was related to lower global DNA methylation in cub and adult hyenas, a finding that requires further investigation and testing of alternative hypotheses regarding the role of social stress.

Given that %CCGG DNA methylation represents coverage in gene bodies and noncoding repetitive sequences of DNA (Ball et al., 2009; Kinney et al., 2011), and that higher methylation of these regions is associated with intragenic exon expression (Li et al., 2018), lower rates of transposon activity (Barau et al., 2016) and genomic stability (Eden et al., 2003; Tuck-Muller et al., 2000), our findings suggest that social and ecological experiences during early life that are associated with lower global DNA methylation may also be determinants of adverse phenotypes or lower fitness in hyenas – a topic for future studies. Furthermore, we recommend longitudinal studies to directly assess the persistence of epigenetic modification over ontogenetic development in long-lived and gregarious species. Finally, incorporating additional information on early life social experience (e.g., maternal care and interactions with peers) should reveal novel insights into how social interactions shape the epigenome (Massart et al., 2017; Provencal et al., 2012; Weaver et al., 2004) in the context of DOHaD.

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DATA ACCESSIBILITY

Data, including independent variables and LUMA DNA methylation values will be archived on GitHub at, https://github.com/laubach/hy_luma. The R analysis code is also stored here and available for public access.

AUTHOR CONTRIBUTIONS

Z.M.L. conceived the research, did the laboratory work and statistical analyses, and wrote the manuscript. K.E.H., D.C.D. and C.D.F. provided financial and intellectual support, helped refine hypotheses and experimental designs, and provided oversight on interpretation of results. L.M.,

T.R.J., and D.R. assisted with laboratory work and provided feedback on the manuscript. M.O.P. darted hyenas and collected field data.

SUPPLEMENTAL INFORMATION

We quantified global DNA methylation from hyena whole blood using the LUminometric Methylation Assay (LUMA) (Karimi, Johansson, & Ekström, 2006; Karimi, Johansson, Stach, et al., 2006). Prior to all LUMA reactions, we used Clean and Concentrate kits (Zymo ®) to remove any EDTA from our DNA samples and eluted in Tris because the chelating action of EDTA can interfere with enzymatic reactions. In paired wells on 96 well plates, ~300 ng of genomic DNA were treated with methyl-sensitive *Hpa*II, and methyl-insensitive *Msp*I restriction enzymes (New England Biolabs®). Both enzymes share sequence specificity, potentially binding at '5-*CCGG*-3' sites across the genome, and actual binding and DNA cleaving depends on the methylation status of 3' cytosine. Both the *HpaII* and *MspI* reactions contained Tango buffer (Thermo Scientific®) with Bovine Serum Albumin (BSA) to optimize enzyme activity as well as *EcoRI* (New England Biolabs®), which ubiquitously cleaves DNA at 5'-*GAATTC*-3' and serves as an inter-sample standardization that accounts for variation in the DNA concentration among samples. We added RNA-free water to each reaction bringing the final volume to 20µL. Using a BioRad C1000 Touch (CT015154) thermal cycler set-up for a 20µL reaction with 105 °C hot lid, we incubated our paired reactions at 37°C for 4hrs followed by a 20min, 80°C enzyme deactivation step.

After restriction enzyme digestion reactions were completed, we prepared samples for pyrosequencing on a Pyromark ® Q96 MD (Qiagen) in order to quantify the proportion of methylated '5-*CCGG*-3' sites in each hyena's genome. We created a LUMA run profile using

PyroMark software (Qiagen) in SNP mode and specified the nucleotide sequence dispensation as *GTGTCACATGTGTG*. Here, it is worth noting that we included two additional GT nucleotide pair dispensations at the beginning of the sequence because these allow nucleotides to fill non-specific overhangs that match the expected overhang from our enzyme digestion, and thus, provide a metric for determining the extent to which an original DNA sample was degraded (T. H. Bjornsson et al., 2008; Head et al., 2014; Sant, Nahar, & Dolinoy, 2012). Before pyrosequencing, we added 20µL annealing buffer (Qiagen®) to both *HpaII* and *MspI* reactions for each sample and transferred 12µL of this mixture into 96-well pyrosequencing plates. All samples were pyrosequenced in duplicate. Following pyrosequencing, we exported the pyrogram peak height data (measured as light units) corresponding to nucleotide incorporation at each dispensation site for each well in our plate, and then used the following equation to calculate percent global methylation at '5-*CCGG*-3' sites across the hyena genome.

$$1 - \frac{(\text{ Disp. } 10\ G\ \text{peak}_{\boldsymbol{HpaII}} / \text{Disp. } 9\ T\ \text{peak}_{\boldsymbol{EcoRI}})}{(\text{ Disp. } 10\ G\ \text{peak}_{\boldsymbol{MspI}} / \text{Disp. } 9\ T\ \text{peak}_{\boldsymbol{EcoRI}})} \times 100$$

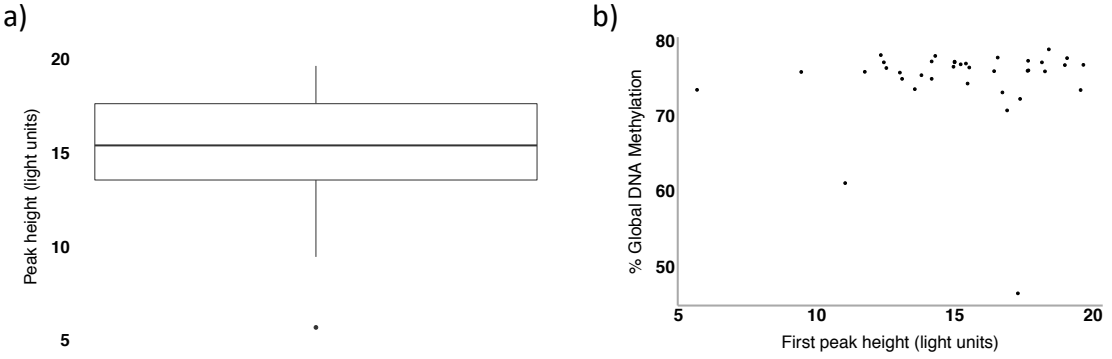
As shown in the equation, first we normalized the *HpaII* and *MspI* enzymatic activity against *EcoRI* by taking the peak height ratio for dispensation 10 over dispensation 9. Next, we divided the normalized *HpaII* peak ratio, which corresponds to DNA cleavage at all '5-*CCGG*-3' sites, by the normalized *MspI* peak ratio, which corresponds to DNA cleavage at unmethylated '5-*CCGG*-3' sites only, and then subtract this value from one to obtain the proportion of methylated '5-*CCGG*-3' sites throughout the whole genome.

Enzymatic digestion of DNA and pyrosequencing are sensitive laboratory techniques that are subject to quality of DNA and slight variations in the reaction conditions between runs

and even within runs (Head et al., 2014), so we used strict quality assessment, quality control (QAQC) to minimize variation due to procedural errors. On all 96 well plates we included no template controls (NTC) for both *HpaII* and *MspI* reactions in which DNA was replaced with nuclease-free water. We used NTC to estimate the lower detection limit of pyrosequencing, and also to assess for potential plate contamination. Our average NTC peak height was 1.68285714 light units. Based on a signal to noise ratio of 5 and our average NTC peak heights (i.e. noise), we calculated a minimal signal peak height for nucleotide incorporation in the presence of DNA as 8.4142857 light units. Therefore, any dispensation with a peak height greater than 8.41 we considered signal and smaller peaks were considered noise. To be conservative we rounded the minimal signal height to 10 light units and set the upper bound of noise as 5 light units. To assess for plate contamination, we determined whether or not there were peaks greater than 5 lights units in our NTC, and a plate was considered contaminated if both pyrosequencing duplicates had peaks greater than 5 at dispensation 9 or 10 for NTC. No plates used in our analyses were contaminated according to this definition. Similarly, we retained for analyses all samples containing DNA if they had peak heights greater than 10 at dispensation 9 and 10. If one of the two duplicates failed due to low peak heights, then we used only the technical duplicate that passed and if both duplicates failed, we re-ran the sample starting with new restriction enzyme reactions.

When running LUMA, fragmented and degraded DNA can cause nucleotide incorporation at non-specific, single strand overhangs in the DNA. This can result in non-specific peaks in the pyrogram and contribute to an artificially enhanced signal at the MspI/HpaII cleavage site, which would have the effect of decreasing % methylation (Head et al., 2014).

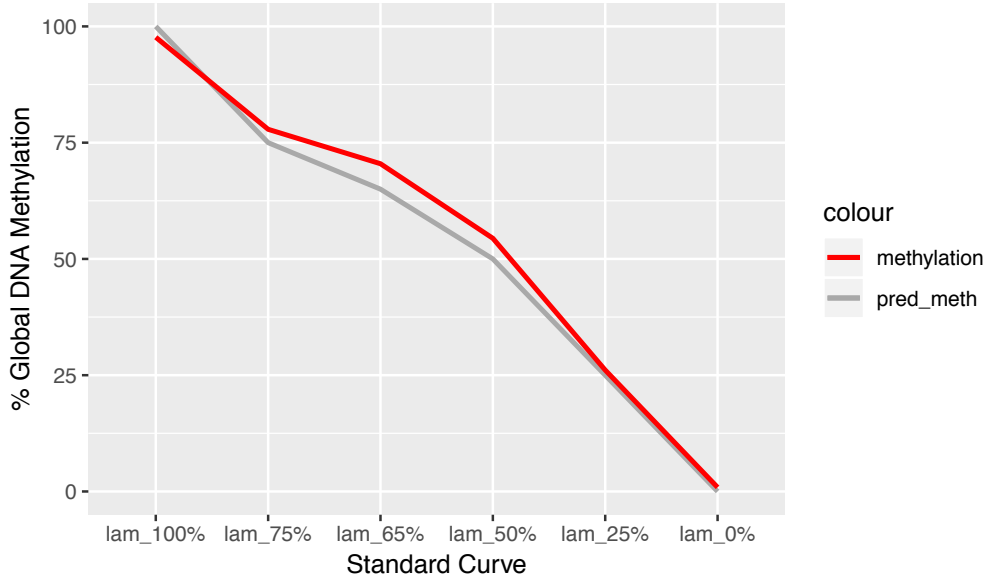
Given the sensitivity of this assay to DNA quality, two approaches have been proposed in the literature. First, Bjornson et al. modified the LUMA protocol by extending the sequence to analyze with additional nucleotide dispensations in order to fill non-specific overhangs in degraded DNA (T. H. Bjornsson et al., 2008). We also used a modified LUMA protocol that included extra nucleotide dispensation as described above. Second, Head et al. suggest excluding any samples for which non-specific peaks are observed in the pyrogram (Head et al., 2014). In our hyena samples we observed an initial non-specific peak (>5 light units) at the first G nucleotide dispensation (Supplemental Figure 3.1a). However, when we graphed % methylation by the peak height of the first G dispensation for the hyena samples on our initial LUMA plate, there was no apparent relationship (Supplemental Figure 3.1b). Recall, we would expect a negative relationship between non-specific peak height and % methylation with degraded DNA.



Supplemental Figure 3.1. Plots characterizing the first G dispensation peak height and %CCGG methylation for hyena samples from the first LUMA plate and pyrosequencing reaction. a) A boxplot of the of first G dispensation peak height. b) A scatterplot of %CCGG methylation by peak height of the first G dispensation.

We also assessed peak heights at dispensations 3, 4, 7, and 8 to determine the quality of the original DNA sample. Peak heights of greater than 5 light units at these positions indicate

incorporation of nucleotides at non-specific DNA overhangs, an indicator that the original DNA sample was fragmented and degraded, and as such we marked the sample as failed. If only one duplicate failed, we retained the sample that did not have non-specific nucleotide incorporation, and if both duplicates failed, we re-ran the sample. Samples that had coefficient of variation (CV) between duplicate percent methylation values that was greater than 5% failed our QAQC and were re-run. As an additional filter to avoid including degraded DNA samples in our analyses, we excluded data that exceeded 2 standard deviations from the mean of the overall % CCGG methylation distribution. This exclusion criteria had the effect of removing some very low methylation values from our final data set that were likely from low quality DNA samples. As a final assessment of our LUMA reaction accuracy, we spiked unmethylated lambda phage DNA in order to create an assay linearization of 0%, 25%, 50%, 60%, 75%, and 100% methylation. The predicted versus observed data did not show any concerning deviations from linearity (Supplemental Figure 3.2).



Supplemental Figure 3.2. Linearization of predicted versus observed methylation values from spiked lambda phage DNA.

Within and between plates, we assessed for variation in global methylation potentially resulting from batch effects of enzymes or procedural error. On every plate we included a hyena pool sample in the top left, middle and bottom right wells. In 1-2 wells on every plate, we also included a hyena pool sample that was spiked to an approximate 100% methylation using Zymo ® CpG Methylase kit. We used these samples to calculate an inter-assay CV of 4.3 and 3.0 for the pool and the 100% spike, respectively. Our intra-plate CV was calculated based on our hyena pool for each of 14 reaction plates and the average intra-plate CV for all 14 plates was 2.3 (range 0.2-5.2). Finally, we selected 43 samples from each of the original 4 LUMA reaction plates (9-12 samples were selected from roughly equal spatial distributions across each reaction plate) and we reran the LUMA assay on these same samples approximately 1.5 years later. The average difference in percent methylation between the original and the rerun samples was 1.85%.

We used the hyena pools and their position on the plate as well as the temporal order in which DNA was added to the reaction wells to calculate a plate-by-plate drift coefficient. As soon as DNA is exposed to restriction enzymes, some enzymatic activity is expected to occur, so DNA digestion should begin earliest in plate wells that receive DNA first. Furthermore, thermal cycler temperature regulation may not be exactly uniform, so in addition to the above-mentioned temporal variability in LUMA reactions, uneven heating and cooling could potentially contribute to spatial bias on reaction plates. Both spatial and temporal variation in enzymatic activity could potentially influence our estimates of global DNA methylation. To account for these potential biases, we assessed a spatio-temporal drift effect on each plate. To do this we regressed the pooled hyena % methylation against the order in which pooled hyena

sample was added to the reaction well and extracted a drift coefficient (β estimate) for each plate. We then used the calibration equations (Supplemental Figure 3.3) to calculate a proportional time weighted methylation value for each sample. In this equation we added or subtracted the proportionally weighted drift estimate from each plate to the raw methylation values in order to calculate an adjusted methylation value. The average difference between each sample's raw and adjusted methylated values was only 0.008%, so in these analyses we used the unadjusted raw values.

	1	2	3	4	5	6	7	8	9	10	11	12
A (MspI)	hy_pool	hy_100%	lamb_100	lamb_75	lamb_65	lamb_50	lamb_25	lamb_0	419	420	424	NTC
B (HpaII)	hy_pool	hy_100%	lamb_100	lamb_75	lamb_65	lamb_50	lamb_25	lamb_0	419	420	424	NTC
C (MspI)	430	432	433	434	439	440	453	454	457	458	462	467
D (HpaII)	430	432	433	434	439	440	453	454	457	458	462	467
E (MspI)	468	469	472	476	484	hy_pool	499	505	506	507	508	509
F (HpaII)	468	469	472	476	484	hy_pool	499	505	506	507	508	509
G (MspI)	513	515	516	520	522	525	529	531	535	536	hy_100%	hy_pool
H (HpaII)	513	515	516	520	522	525	529	531	535	536	hy_100%	hy_pool

$$\text{Raw methylation}_{pos} + \left(\left(1 - \frac{n_{pos}}{30}\right) \times \beta_{plate}\right) = \text{Adjusted methylation}_{pos}$$

$$\text{Raw methylation}_{pos} - \left(\left(\frac{n_{pos}}{30} - 1\right) \times \beta_{plate}\right) = \text{Adjusted methylation}_{pos}$$

Supplemental Figure 3.3. Calibration equations and the basic plate layout for calculating an adjusted methylation value based on a plate specific drift coefficient (β_{plate} estimate) that is proportionally time weighted according to the order in which DNA template is added to each reaction well. Blue tinted wells have weighted drift value added to raw methylation value, while red tinted well have weighted drift value subtracted. Most plate specific drift coefficients (β_{plate}) were negative.

	β (95% CI)	P-value
MAIN EFFECTS		
Age group for Q1 of maternal rank only		
Cubs	0.00 (Reference)	--
Sub-adults	**4.18 (2.27, 6.08)**	**0.0002**
Adults	**4.10 (1.85, 6.35)**	**0.0013**
Maternal rank (for cubs only)		
Q1 (Lowest)	**0.00 (Refernece)**	**--**
Q2	**3.73 (1.73, 5.73)**	**0.0003**
Q3	**3.83 (1.81, 5.85)**	**0.0002**
Q4 (Highest)	**2.17 (0.04, 4.30)**	**0.0457**
Sex for Q1 of maternal rank and cubs only		
Female	0.00 (Reference)	--
Male	-0.12 (-0.85, 0.60)	0.7362
INTERACTION EFFECTS FOR AGE_GROUP*MATERNAL_RANK		
Sub-adult age group/Maternal rank Q2 - Q1 vs. Cub age group/Maternal rank Q2 - Q1 (reference)	**-4.19 (-6.75, -1.63)**	**0.0031**
Adult age group/Maternal rank Q2 - Q1 vs. Cub age group/Maternal rank Q2 - Q1 (reference)	**-3.16 (-6.15, -0.16)**	**0.0399**
Sub-adult age group/Maternal rank Q3 - Q1 vs. Cub age group/Maternal rank Q3 - Q1 (reference)	**-4.33 (-6.93, -1.73)**	**0.0026**
Adult age group/Maternak rank Q3- Q1 vs. Cub age group/Maternal rank Q3 - Q1 (reference)	**-3.38 (-6.38, -0.38)**	**0.0296**
Sub-adult age group/Maternal rank Q4 - Q1 vs. Cub age group/Maternal rank Q4 - Q1 (reference)	**-2.95 (-5.67, -0.23)**	**0.0350**
Adult age group/Maternal rank Q4 - Q1 vs. Cub age group/Maternal rank Q4 - Q1 (reference)	*-2.61 (-5.55, 0.33)*	*0.0788*

In this model, %CCGG methylation is the outcome and covariates include: offspring categorical age, sex, maternal rank the year an offspring was born, a maternal rank by categorical age interaction term, and a random intercept for offspring ID.
Bold estimates are significant at p<0.05, and italicized estimates are significant at p<0.1

Supplemental Table 3.1. An interaction model and adjusted associations of explanatory variables with global DNA methylation for all age groups.

	β (95% CI) for %CCGG methylation					
	Cub models	P	Subadutl models	P	Adult models	P
EARLY LIFE SOCIAL ENVIRONMENT						
Maternal rank during birth year						
Q1 (Lowest)	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Q2	**3.21 (0.79, 5.63)**	**0.012**	-0.50 (-1.77, 0.77)	0.440	0.54 (-0.87, 1.95)	0.453
Q3	**3.33 (0.88, 5.78)**	**0.010**	-0.52 (-1.83, 0.78)	0.436	0.52 (-0.89, 1.93)	0.473
Q4 (Highest)	1.61 (-1.00, 4.23)	0.232	-0.82 (-2.13 0.50)	0.228	-0.40 (-1.68, 0.89)	0.548
Litter size						
Singleton	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Twins	0.71 (-1.47, 2.90)	0.525	-1.06 (-2.44, 0.33)	0.139	-0.03 (-1.45, 1.39)	0.967
ECOLOGICAL FACTORS						
Anthropogenic disturbance during birth year						
Low	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Medium	**2.74 (0.87, 4.61)**	**0.006**	0.82 (-0.46, 2.09)	0.214	0.61 (-0.42, 1.64)	0.251
High	**3.41 (0.72, 6.09)**	**0.016**	**2.03 (0.84, 3.21)**	**0.001**	*1.14 (-0.04, 2.32)*	*0.062*
Prey density during discrete developmental periods (per 1 SD)[†,‡]						
Periconception	**-1.24 (-2.04, -0.43)**	**0.004**	0.04 (-0.53, 0.60)	0.899	-0.02 (-0.50, 0.46)	0.937
Gestation	*-0.66 (-1.40, 0.08)*	*0.086*	0.30 (-0.55, 1.15)	0.494	-0.12 (-0.54, 0.29)	0.558
Birth to <3 months of age	**-1.55 (-2.58, -0.53)**	**0.004**	-0.36 (-0.89, 0.17)	0.186	**-0.50 (-0.90, -0.09)**	**0.019**
3 to <6 months of age	-0.09 (-1.17, 1.00)	0.875	0.23 (-0.25, 0.71)	0.347	-0.06 (-0.62, 0.49)	0.826
6 to <9 months of age	-0.08 (-0.94, 0.78)	0.852	0.09 (-0.53, 0.71)	0.782	-0.16 (-0.63, 0.31)	0.506

[†] Prey species include the 5 most commonly consumed wild ungulates: Impala, Thomson's gazelle, Topi, Plains zebra, and Wildebeest.
[‡] Models are adjusted for prey densities from previous developmental periods.
Bold estimates are significant at p<0.05, and italicized estimates are significant at p<0.1

Supplemental Table 3.2. Unadjusted associations of explanatory variables with global DNA methylation in hyenas.

	β (95% CI) for %CCGG methylation			
	Own rank excluded	P	Own rank included	p
Anthropogenic disturbance during birth year				
Maternal rank during birth year				
Q1 (Lowest)	0.00 (Reference)		0.00 (Reference)	
Q2	0.98 (-0.97, 2.94)	0.331	*2.37 (-0.26, 5.01)*	*0.087*
Q3	0.52 (-1.28, 2.31)	0.576	1.14 (-1.14, 3.43)	0.333
Q4 (Highest)	-0.77 (-2.57, 1.02)	0.404	-1.16 (-4.09, 1.78)	0.446
Hyenas rank during year when darted				
Q1 (Lowest)	NA		0.00 (Reference)	
Q2	NA		1.66 (-0.47, 3.78)	0.136
Q3	NA		1.48 (-1.27, 4.23)	0.299
Q4 (Highest)	NA		1.29 (-2.12, 4.70)	0.464

Models are adjusted for hyena age at darting (months) and include only adult females.

VIF for birth year maternal rank and a hyena's own rank the year it was darted in the model where both variables were included were 6.82 and 7.41, respectively.

Bold estimates are significant at p<0.05, and italicized estimates are significant at p<0.1

Supplemental Table 3.3. Sensitivity analysis in which models both exclude and include an adult hyena's own rank the year it was darted, as well its mom's rank the year it was born.

	β (95% CI) for %CCGG methylation					
	Cub	P	Subadult	P	Adult	P
Sample age						
Year sample was collected	**0.25 (0.09, 0.43)**	**0.004**	**0.15 (0.08, 0.23)**	**<0.001**	0.05 (-0.03, 0.13)	0.197
Anthropogenic disturbance during birth year						
Not adjusted for sample age						
Low	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Medium	**2.88 (0.99, 4.78)**	**0.004**	0.84 (-0.44, 2.13)	0.201	0.54 (-0.56, 1.63)	0.340
High	**3.51 (0.82, 6.19)**	**0.013**	**2.05 (0.85, 3.25)**	**0.001**	0.80 (-0.49, 2.09)	0.229
Adjusted for sample age						
Low	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Medium	1.67 (-0.98, 4.31)	0.221	-0.31 (-2.10, 1.48)	0.735	0.43 (-0.93, 1.79)	0.537
High	-0.08 (-6.17, 6.02)	0.980	-0.25 (-3.02, 2.53)	0.862	0.64 (-1.14, 2.42)	0.483
Maternal rank during birth year						
Not adjusted for sample age						
Q1 (Lowest)	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Q2	**3.19 (0.68, 5.71)**	**0.016**	-0.50 (-1.78, 0.78	0.444	0.52 (-0.85, 1.88)	0.461
Q3	**3.46 (0.96, 5.97)**	**0.009**	-0.47 (-1.78, 0.85)	0.486	0.81 (-0.59, 2.21)	0.261
Q4 (Highest)	1.68 (-0.96, 4.32)	0.217	-0.72 (-2.06, 0.62)	0.296	-0.64 (-1.94, 0.65)	0.333
Adjusted for sample age						
Q1 (Lowest)	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Q2	**2.84 (0.43, 5.25)**	**0.025**	-0.70 (-1.90, 0.50)	0.252	0.60 (-0.79, 1.99)	0.400
Q3	**3.17 (0.77, 5.57)**	**0.012**	-0.73 (-1.96, 0.50)	0.248	0.80 (-0.61, 2.21)	0.269
Q4 (Highest)	1.77 (-0.75, 4.29)	0.174	-1.02 (-2.36, 0.16)	0.091	-0.63 (-1.93, 0.67)	0.345

Models are adjusted for hyena age at blood collection (months) and sex.

Bold estimates are significant at p<0.05, and italicized estimates are significant at p<0.1

Supplemental Table 3.4. Sensitivity analysis in which models both exclude and include sample age as a covariate. Associations between anthropogenic disturbance and maternal rank with global DNA methylation in hyena cubs, subadults and adults are reported.

CHAPTER 4

ASSOCIATIONS OF MATERNAL CARE AND EARLY LIFE SOCIAL EXPERIENCE WITH OFFSPRING DNA METHYLATION AND LATER LIFE STRESS PHENOTYPE

Zachary M. Laubach, Julia R. Greenberg, Julie W. Turner, Christopher D. Faulk, Dana C. Dolinoy, Tracy Montgomery, Laura Smale, Wei Perng, Elise Zipkin and Kay E. Holekamp

INTRODUCTION

Social experiences, particularly early in life, affect the behaviors and physiology of organisms. Longstanding evidence dating back to the 1950s and 60s demonstrates that maternal care and interactions with peers affect hypothalamic pituitary adrenal (HPA) axis function and development of stress-related phenotypes in nonhuman primates (H F Harlow, Dodsworth, & Harlow, 1965; Harry F. Harlow & Zimmermann, 1958; Sanchez, 2006; Stephen J . Suomi, Harlow, & Novak, 1974). Similarly, rodent studies have found that maternal neglect (e.g., lower rates of maternal licking and grooming) during the offspring's first ten days of life is a determinant of elevated adult plasma corticosterone in response to external stressors (Liu et al., 1997), and greater exhibition of fearfulness behaviors (Caldji et al., 1998; Francis, Diorio, Liu, & Meaney, 1999). In humans, social deprivation of children who lived in orphanages is associated with altered HPA activity, including elevated basal cortisol levels that were evident even after adoption (Fries, Shirtcliff, & Pollak, 2008). Similarly, Carpenter et al. found that among 230 healthy adult humans who passed a psychological and physical prescreening examination, self-reported feelings of being unwanted early in life corresponded with a blunted cortisol response to a standardized hormonal stimulus (Carpenter et al., 2009). Taken together,

evidence from diverse mammalian taxa point toward the importance of maternal care and early life social interactions to the development of a stress phenotype. A persistent question that remains is *how* early social experiences affect stress phenotypes and health.

One mechanism proposed to underlie the relationship between adverse early social experiences and poor stress phenotypes later in life is DNA methylation, a mitotically stable but modifiable epigenetic mark that is responsive to environmental cues and is associated with regulation of gene expression (Klose & Bird, 2006; E. Li & Bird, 2007). A landmark cross-fostering study in rodents showed that higher rates of maternal licking and grooming were associated with higher methylation of the promoter DNA region of the hippocampal Glucocorticoid Receptor (GR; *NR3C1*) gene, lower GR RNA expression, and elevated plasma corticosterone among adult rat offspring (Weaver et al., 2004). Subsequent work in rodents demonstrated that maternal care behaviors also induced widespread differences in DNA methylation across the entire genome – not only at single gene promoter regions (Anier et al., 2014; McGowan et al., 2011). In rhesus macaques, rearing conditions (maternal vs peer-rearing), corresponded with genome-wide changes in DNA methylation assessed in both brain and T-cells during adulthood (Provencal et al., 2012). In parallel with findings from animal models, epidemiological studies in humans reported that lower self-reported maternal care corresponded to higher DNA methylation of two stress related genes during adulthood (Unternaehrer et al., 2015). Taken together, these studies suggest that social experience during early life not only has a marked effect on one's future stress phenotype, but also, that patterns of DNA methylation and RNA expression, particularly involving the GR gene, may mediate this relationship.

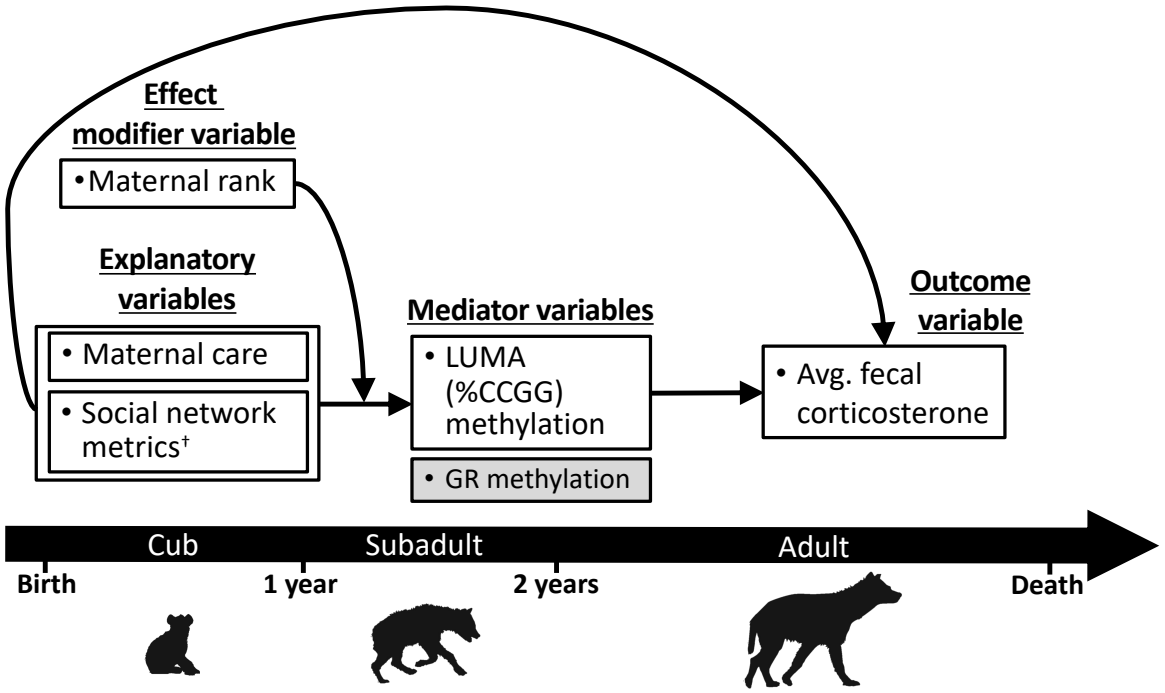
There are three lacunae in current knowledge of how early social experiences shape later stress phenotypes. First, despite the plethora of molecular and behavioral evidence from laboratory rodents (Szyf, Weaver, Champagne, Diorio, & Meaney, 2005; Weaver et al., 2004, 2005), this model lacks social complexity and genetic diversity for extrapolation to gregarious animal species in their natural habitats. Although non-human primates represent an improvement in regard to social complexity and genetic diversity (Massart et al., 2014; Provencal et al., 2012), such studies have relied on rather extreme early life exposures (e.g. maternal separation) that likely fail to capture the nuanced effects of naturally occurring variation in maternal care and other social interactions. Finally, in light of the dynamic nature of growth and development as well as the responsive nature of epigenetic mechanisms to environmental stimuli, it is likely that the effect of early social experiences on DNA methylation and stress phenotype may vary over the course of ontogeny. Therefore, explicit consideration of the timing of social experiences in relation to relevant biological pathways and stress outcomes is critical for a deeper etiological understanding.

Here, we examine the relationships among early social experience, DNA methylation, and stress phenotype in an ongoing field study of a wild population of spotted hyenas (*Crocuta crocuta*) living in the Masai Mara National Reserve, Kenya. These social mammals represent a novel and relevant system within which to explore our research questions, as they exhibit a wide range of social behaviors and live in large fission-fusion groups, called “clans,” which can contain more than 100 individuals (Green et al., 2018; Kruuk, 1972). Of particular relevance to our research interests is the fact that a key subset of hyena social behaviors involves mothers and their offspring. Female hyenas reach reproductive maturity around two years of age and

typically give birth to 1 or 2 offspring every 14 to 17 months throughout their lives, starting in their third year of life (Holekamp, Smale, & Szykman, 1996). Following a 110-day gestation period, hyena cubs are born in a natal den and spend the first three to four weeks of life interacting exclusively with their mothers (Holekamp & Smale, 1998). Due to their protracted development, hyena offspring rely on their mothers for sustenance and social support until they are at least two years of age (Holekamp & Smale, 1998). In addition to interacting with their mothers, young hyenas socialize with other members of their clan starting when their mothers bring them to the clan's communal den. After a period of residing predominantly in the communal den, den independent hyenas venture out into their clan's territory where they further develop their social networks (Turner, Bills, & Holekamp, 2018). During this stage of development, den independent subadult hyenas play a more active role in deciding with which members of their group they associate and interact with on a regular basis (Holekamp et al., 1997; Turner et al., 2018; Wahaj et al., 2004). The lengthy period of maternal care, coupled with the hyena's complex social milieu, offer a compelling system in which to test hypotheses investigating the extent to which early social experiences are associated with molecular biomarkers, including DNA methylation, as well as with health outcomes, such as stress phenotype in adulthood.

The objectives of the present study are threefold, following the steps of a standard mediation analysis. First, we sought to characterize the relationship between the explanatory variables of interest (early life social environment as indicated by maternal care and social network metrics) with our outcome of interest (adult fecal corticosterone concentrations.) Next, we examined associations between the explanatory variables and two metrics of DNA

methylation (global DNA methylation and gene-specific methylation of the glucocorticoid receptor region), and between DNA methylation and the adult stress phenotype. Finally, assuming that results from the first two steps supported the notion that DNA methylation might provide a link between explanatory variables and the outcome, we assessed the extent of mediation by DNA methylation (Figure 4.1).



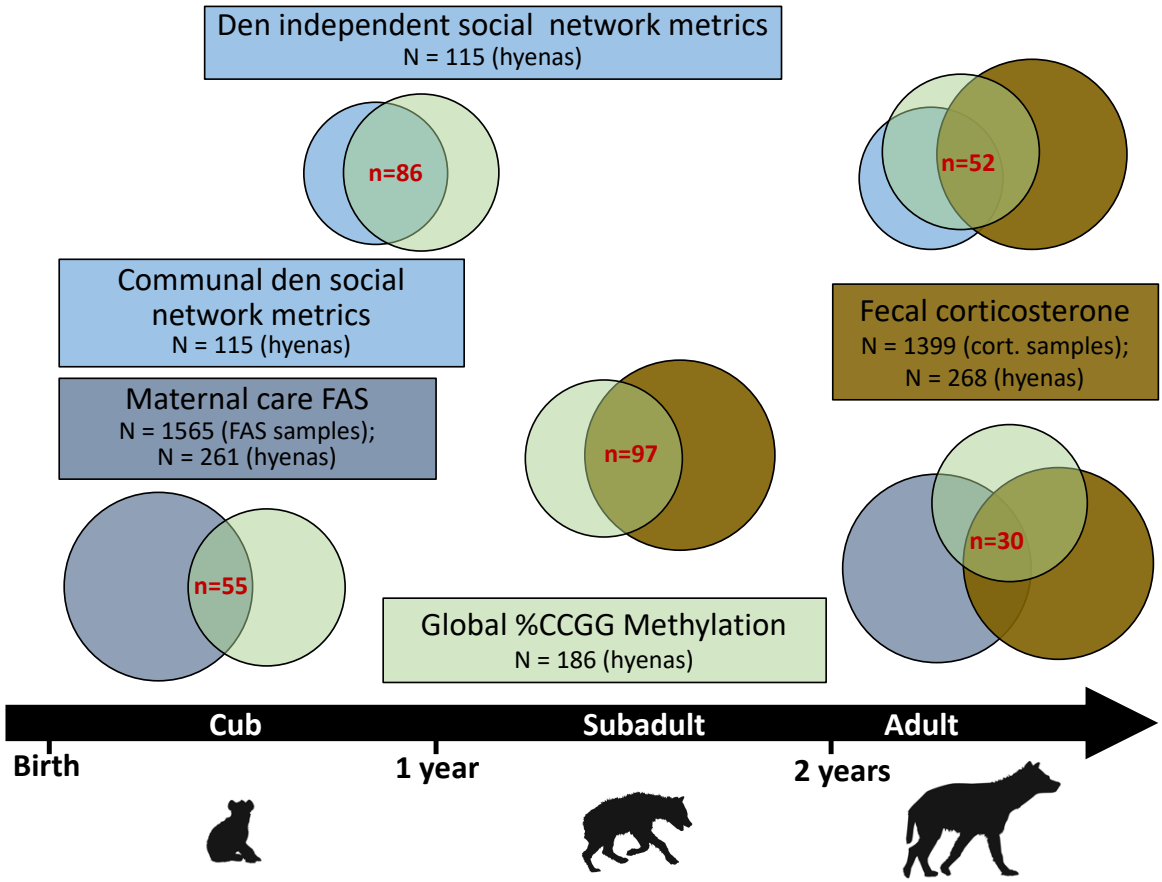
† Social network metrics were assessed both during communal den (CD) and den independent (DI) periods of development.
Edges of boxes only roughly correspond to the hyenas' ages when samples were collected.

Figure 4.1 Directed Acyclic Graph (DAG) showing an overview of the conceptual framework and hypotheses being tested relating early life maternal care and social network connections to DNA methylation and adult stress phenotype. Maternal rank is posited to affect the relationship between early life social factors and DNA methylation. Glucocorticoid Receptor (GR) DNA methylation was excluded from downstream analyses due to low (between 0-1%) variation in CpG site methylation (see Supplemental material).

METHODS

Study population

We used behavioral data and biological samples from the Mara Hyena Project, collected between June 1988 and July 2016 from wild spotted hyenas in Kenya. The subset of data used in our analyses comprises four overlapping data sets with information on: (1) DNA methylation measures from 186 cub and subadult hyenas (age ≤24 months), (2) maternal care behaviors from focal animal survey (FAS) data on 261 unique mother-cub pairs when cubs were less than 13 months old, which is the approximate age at weaning (Holekamp, K. E., & Smale, L., 1998), (3) social network data from 115 hyenas during two early phases of development: the communal den (CD) period when a young hyena resided exclusively at the communal den, and the den independent (DI) period, which began when cubs were found away from the communal den on at least 4 consecutive occasions, and (4) fecal corticosterone measures from 268 adult (>24 months old) hyenas. The overlap among data sets, and the final analytical samples sizes, are shown in Figure 4.2.



Edges of boxes roughly correspond to the hyenas' ages when samples were collected.

Figure 4.2. Overview of the sampling design and sample sizes from the four data sets used in these analyses. The timeline on the bottom shows the different life stages of hyenas. Each color of box represents a distinct type of data collected from our study population. Sample size and numbers of individual hyenas after data cleaning are indicated inside each box. Venn diagrams show the overlap in sample size among the different data sets.

Our final sample was restricted to mother-offspring pairs for which the earliest date of the maternal care FAS session and the start date of the social network period preceded the immobilization date when blood was drawn, and DNA methylation was measured. We also included only fecal corticosterone measures that were obtained after assessments of maternal care behaviors, social network metrics, and %CCGG DNA methylation in order to preserve

temporal relationships between our explanatory variables and our outcome variables to improve causal inference.

For each of the hyenas in our study population, we also have information on a number of biological, social experience, and ecological variables. We estimated each hyena's age with an accuracy ± 7 days based on the behaviors and morphology of each cub when they were first observed outside of their natal den (Holekamp et al., 1996). Once a hyena was three months old, we determined their sex based on the glans morphology of their erect phallus (Frank, Glickman, & Powch, 1990). Maternal rank the year each offspring was born was calculated based on each adult female's wins and losses during agonistic interactions for a given year (Engh et al., 2000; Holekamp & Smale, 1993; Smale et al., 1993). We normalized adult female ranks each year on a scale of -1 (lowest rank) to 1 (highest rank) to account for changes in group size. We identified if young hyenas had a sibling or not and grouped them as twins or singletons, respectively. Using our detailed demographic data, we recorded each mom's parity for a given offspring and categorized this variable as either primiparous or multiparous. We also recorded clan size over the duration of our study period. Next, we categorized offspring hyenas into groups according to whether they were born in low, medium, or high human disturbance based on illegal livestock grazing in the park (Green et al., 2018). We also grouped them into two categories of food availability based on the time of year that they were born. Birth dates roughly corresponding to the annual wildebeest and zebra migration from June – November were categorized as migration present and hyenas born December- May were categorized as migration absent. Collectively, age and sex were denoted as our 'biological confounding variables,' maternal rank, litter size, parity and clan size were included in our 'social experience

confounding variables,' and human disturbance and migration status were grouped in the 'ecological confounding variables.' In the following section, we describe each of the four source datasets.

Data set #1: DNA methylation measures from 186 cub and subadult hyenas

Blood collection, processing, and storage

Hyenas from our study population were immobilized using a CO_2 rifle that propelled a pressurized dart containing 6.5 mg/kg of tiletamine-zolazepam (Telazol ®). Within 13 minutes of when the hyena was sedated, we drew blood from their jugular vein into ethylenediaminetetraacetic acid (EDTA) coated vacuum tubes. We flash froze whole blood samples in liquid nitrogen or we extracted genomic DNA using Gentra Pure Gene kits by Qiagen® and stored samples until they were transported to the U.S. for long term storage at -80℃.

DNA methylation assays

We quantified global DNA methylation derived from whole blood using the LUminometric Methylation Assay (LUMA) (Karimi, Johansson, & Ekström, 2006; Karimi et al., 2006). A description of LUMA, the laboratory procedure, and our data cleaning protocol are described in detail in (Laubach, Faulk, et al., 2019). In brief, this assay uses a parallel enzyme digestion of DNA to quantify the amount of methylated vs unmethylated CpG sites within the '5-CCGG-3' recognition sequence. Following pyrosequencing of the enzyme-digested DNA, we calculated a composite global methylation value for each hyena representing the average DNA

methylation across the hyena genome. As we and others have previously described, there are approximately 2.4 million CCGG motifs in the mammalian genome, and based upon the high resolution human genome, we expected about 3% of the CCGG motifs to occur within 1kb of transcription start sites, 45% in gene bodies and 52% in non-coding regions of the genome (Ball et al., 2009; Kinney et al., 2011; Laubach, Faulk, et al., 2019). The vast majority of CpG sites assessed via LUMA occur in gene bodies, where they may function in transcription regulation and alternative splicing (Maor, L., Yearim, & Ast, 2015; Li, Zhang, Huang, & He, 2018), as well as in non-coding regions of genome, where they may repress repetitive elements (Barau et al., 2016; Coluccio et al., 2018) and enhance chromosome stability (Eden et al., 2003; Tuck-Muller et al., 2000). Therefore, we made the basic inferential assumption that lower than average %CCGG methylation is likely disadvantageous to health and fitness.

In addition to our global DNA methylation assays, we also assessed CpG methylation in the putative glucocorticoid receptor (GR) promoter region of DNA from 96 hyenas. An overview of our bioinformatic and laboratory methods for this candidate gene approach can be found in the Supplemental material. We identified CpG sites in the hyena genome which overlapped with DNA from humans and rats (Supplemental Figures 4.4 and 4.5) that, in non-hyena species, have been shown to be differentially methylated with respect to early life environment (Mccormick et al., 2000; McGowan et al., 2009; Oberlander et al., 2008; Perroud et al., 2011; Szyf et al., 2005; Weaver et al., 2004). Next, we developed a pyrosequencing assay to quantify site-specific CpG methylation of bisulfite treated DNA. In a pilot sample of 96 hyenas we calculated CpG site-specific DNA methylation in the putative hyena GR promoter region. Due to

the invariant and near zero percent methylation at these CpG sites (Supplemental Figure 4.6), we did not analyze additional hyenas with our GR assay.

Data set #2: Maternal care behaviors from focal animal survey (FAS) data on 261 unique mother-offspring pairs

Maternal care focal animal survey (FAS) data collection

We derived the maternal care variables from a FAS data set that comprised sessions in which: (1) mother-offspring pairs were present together for a minimum of five minutes and offspring were less than 13 months old when the FAS data were collected, and (2) where mothers were lactating since our intention was to focus on maternal care received early in life while offspring were dependent on nursing for sustenance. After filtering the data, we retained 1565 FAS totaling more than 7,000 hours of observation of mother-infant pairs. Information on additional data processing steps to prepare variables for use in statistical analyses are described in the Statistical Methods section.

We quantified maternal care from FAS data (Altmann, 1974) based on counts of behaviors occurring during each minute of observation in which both the mother and offspring were present. Behavioral data were collected twice daily between 0530 – 0900 h and 1700 – 2000 h. We focused on three maternal care behaviors: number of minutes the mother and cub spent in close proximity (≤1 meter) to one another), number of minutes spent nursing, and number of minutes during which mothers were observed grooming (i.e., licking) their offspring.

Data set #3: Social network metrics derived from 115 hyenas during the CD and DI phases

We generated association networks among hyenas; these are undirected social networks that quantify co-occurrences between each hyena and its group mates. Association networks were based on regular 15-20 minute interval scan sampling during observations sessions in which two or more hyenas were present (Turner et al., 2018). The networks incorporated twice weighted association index data (Cairns & Schwager, 1986), which enabled us to correct for sampling bias that could stem from variation in hyena group observability, thus providing more reliable information about social bonds in our population (Holekamp, K.E., Smith, Strelioff, Van Horn, & Watts, 2012; Turner et al., 2018).

For each individual hyena, we constructed multiple association networks during the communal den (CD) and den independent (DI) phases of development. A detailed description of the data processing and building of social networks can be found in Turner *et al.* (2018). Briefly, in the current paper we focused on association networks that utilized data from the CD period, when a young hyena resided exclusively at the communal den, and the DI period, marked by the young hyena making repeated excursions out into its clan's territory, away from the communal den. In order to balance our sampling design, we matched the duration of the DI period with that of the CD period.

From each hyena's association networks during its CD and DI stages, we extracted three metrics that quantify the extent to which the hyena is connected within its group, including degree centrality, strength, and betweenness centrality. In our association networks, degree centrality (i.e., degree) corresponded to the number of different individuals with which the hyena was recorded in the same session. Strength, a weighted metric of degree or network

connectedness (Wey & Blumstein, 2012), corresponded to the total number of times the hyena was observed with other clan mates, including repeated associations with the same individuals. Betweenness centrality (i.e., betweenness) was calculated as the number of shortest paths that connected hyenas in the group and passed through the hyena of interest (Wey & Blumstein, 2012). An individual with high betweenness can be thought of as a bridge in which otherwise unconnected members of the network are indirectly connected. We focused our analyses on these three association network metrics because they directly measure an individual hyena's connectedness within each network rather than other metrics that provide additional information about the overall network structure.

Data set #4: Fecal corticosterone measures from 268 adult (>24 months of age) hyenas

Fecal collection and corticosterone assays

Since January 1993, we have opportunistically collected a fecal sample anytime an individually identified hyena was seen defecating. Fecal samples were mixed, and part of the sample was transferred to 2mL cryovials before flash freezing the sample in liquid Nitrogen. Fecal samples were frozen within 12 h of collection and transported frozen from our field site to our lab in the U.S. In this paper we focus on the stress hormone, corticosterone. A detailed description of the hormone extraction process, from the lyophilization and ethanol extraction to the corticosterone radioimmunoassay, was previously described for our study population by (Dloniak et al., 2004; Van Meter et al., 2009).

Statistical analyses

Data exploration

Prior to formal analysis, we assessed the distribution of continuous variables as well as frequency tabulations of categorical variables to check for deviations from normality, errors in the data, missing values, and sample sizes within strata. We constructed boxplots to identify outliers and viewed scatterplots to check linearity of associations between covariates and our outcome variables. We also examined bivariate associations between dependent and independent variables to help identify potential confounding variables that could influence the relationships of interest in our final models so that they could be included as covariates.

Our FAS data were collected and behaviors extracted in four distinct study periods between 1998 and 2013. Therefore, we checked for consistency between the four periods of data collection by visually inspecting a principle component analysis (PCA) plot (Supplemental Figure 4.7). We saw no evidence of clustering by sample collection period and concluded that variation in maternal care was likely not confounded by sampling batch effects, so we were able to pool data from all study periods together.

Data processing

Given that we had anywhere from 1 to 29 repeated observations of mother-offspring interactions for each maternal behavior of interest (close proximity, nursing, and grooming) for each mother-offspring pair, the first step to processing these data for analysis was to convert these repeated measurements into a single value for use in the regression models. To do this, we fit generalized linear mixed-models where the repeated outcome of interest was counts of

each maternal care behavior for a given mother-offspring pair. Fixed effects covariates included key characteristics having potential to affect mother-offspring interactions: the offspring's age in months on the date of the FAS, timing of the FAS (morning or evening), and FAS season (during the annual wildebeest and zebra migration, present or absent). We also included an offset of the natural log of the length of time the mother-offspring were both present during the FAS to control for observer effort. (NB: the offset also facilitates interpretation of parameter estimates such that they reflect incident rates or proportions of time spent engaged in a particular behavior in relation to the total time the mother-offspring pair was observed.) Our models also included a random intercept for offspring hyena ID to account for correlations among the repeated observations. Using these mixed models, we calculated the Best Linear Unbiased Predictors (BLUPs) for each mother-offspring pair. Because behavioral count data are often over-dispersed and zero-inflated, we fit models assuming three different underlying distributions each with and without a zero-inflation correction for a total of six model specifications per type of maternal care behavior. We used the R package glmmTMB to fit a Poisson distributed model in which the mean equals the variance, as well as two parameterizations of negative binomial distributed models which differ in how the variance scales with respect to the mean (Brooks et al., 2017; Magnusson et al., 2019). More specifically, in the negative binomial 1 models, the variance is scaled as multiplicative function of the mean and an estimated dispersion parameter, while in the negative binomial 2 models the variance is scaled as a quadratic function of the mean and an estimated dispersion parameter (Cameron & Trivedi, 1998). We used a simple form of a zero-inflation correction in which each observation has an equal probability of being a zero (Brooks et al., 2017). We compared model fit using AIC

for each distribution with and without modeling zero-inflation. After selecting the best-fitting model with the lowest AIC (see Table 4.1.), we extracted the individual-level random effect estimates from models in which close proximity, nursing, and grooming were the outcomes.

Maternal Care behaviors	dAIC	Deg. freedom
Close proximity		
Zero-inflated negative binomial (2) distributed model [†,§]	0.0	7
Zero-inflated negative binomial (1) distributed model [†,‡]	22.9	7
Negative binomial (1) distributed model	328.2	6
Negative binomial (2) distributed model	409.2	6
Zero-inflated Poisson distributed model [†]	7144.3	6
Poisson distributed model	10115.7	5
Nursing		
Zero-inflated negative binomial (1) distributed model [†,‡]	0.0	7
Zero-inflated negative binomial (2) distributed model [†,§]	14.4	7
Negative binomial (1) distributed model	781.1	6
Zero-inflated Poisson distributed model	850.7	6
Negative binomial (2) distributed model	1078.0	6
Poisson distributed model	9712.4	5
Grooming		
Zero-inflated negative binomial (1) distributed model [†,‡]	0.0	7
Negative binomial (1) distributed model	13.0	6
Zero-inflated negative binomial (2) distributed model [†,§]	44.0	7
Negative binomial (2) distributed model	45.4	6
Zero-inflated Poisson distributed model [†]	146.1	6
Poisson distributed model	791.8	5

Models are adjusted for offspring hyena's age (months), time of day, and migration season on the date of the FAS. Models include a random intercept for offspring ID.

[†] Single zero inflation parameter applied to all observations.

[‡] Negative binomial 1 where, variance = $(1+\alpha)\mu$.

[§] Negative binomial 2 where, (variance = $\mu + \alpha\mu^2$

Table 4.1. AIC values for generalized linear mixed models and zero-inflated generalized linear mixed-models of maternal care behaviors. The best fitting model (lowest AIC) was used to generate Best Unbiased Linear Predictors (BLUPs) for each hyena offspring.

These individual-level random effect estimates represent the underlying distribution of deviation of each maternal care behavior received by each hyena in comparison to the population average for that particular behavior, while controlling for variation in offspring age, FAS time of day and FAS migration season. We then added the individual random effects to the overall model intercept, which represents a relevant population-level biological anchor (i.e., the average amount of maternal care received by the overall population when all other variables are set to the referent level). After appending the BLUPs to the intercept, we exponentiated the variable to transform the estimates from the natural log scale back to the original scale, proportion of minutes during which the mother-infant pair were observed together. Finally, we z-score standardized all maternal care BLUPs and all association index social network metrics so that, when included as explanatory variables in regression analyses, the estimates would be comparable to one another.

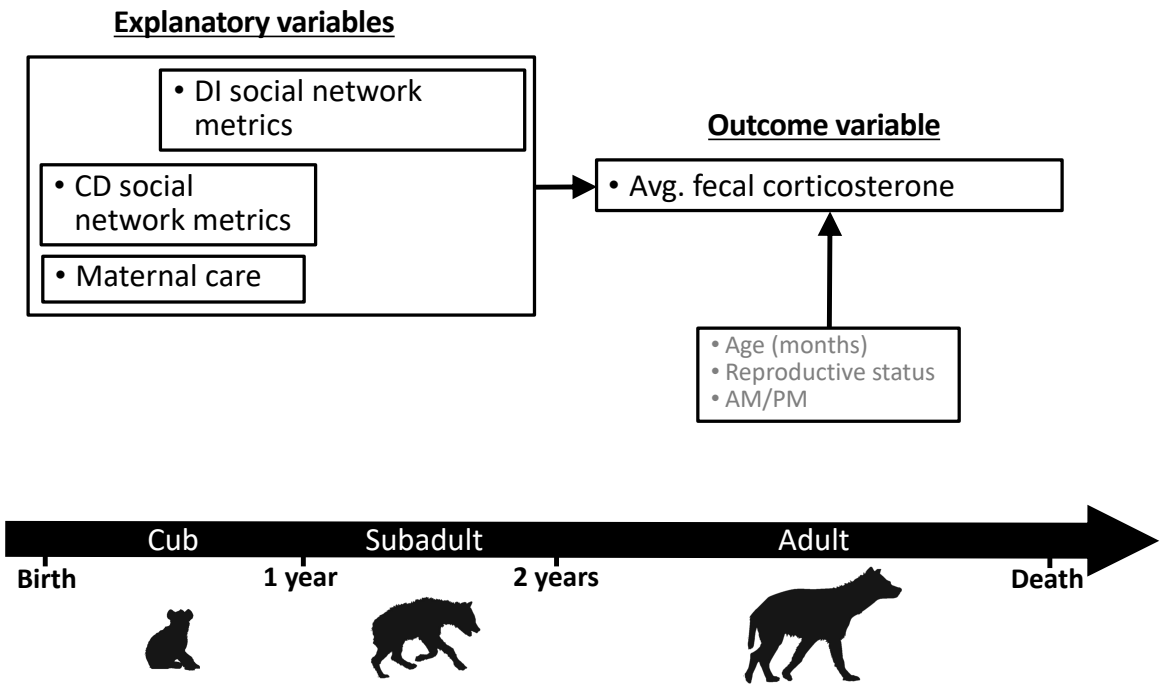
Main analysis

We carried out the main analysis in three distinct steps, conforming to our study aims and following Baron & Kenny's (1986) framework for mediation analysis. First, we sought to characterize the relationship between the explanatory variables of interest (early life social environment as indicated by maternal care and social network metrics) and adult stress phenotype (adult fecal corticosterone concentrations). Next, we examined associations between the explanatory variables and DNA methylation, and between DNA methylation and fecal corticosterone. Finally, assuming that the first two steps indicated that DNA methylation might link the relationship between the early life social environment and the adult stress

phenotype (i.e., fecal corticosterone), our third step was to formally examine mediation by DNA methylation using regression models that included both the early social environment and DNA methylation variables of interest in order to compare the association of early social environment with adult stress phenotype before and after inclusion of the DNA methylation metric.

Step 1: Associations of early life social environment with adult fecal corticosterone (explanatory variable → outcome)

Using linear mixed regression models, we assessed the associations between each maternal care and social network metric as explanatory variables with corticosterone concentration as our outcome. In these models we controlled for precision covariates, including a hyena's age, its reproductive state / sex (nulliparous, pregnant, lactating, other, or male), and the time of day (A.M. or P.M) when the fecal sample was collected. Covariates were selected based upon *a priori* biological knowledge from previous studies in this species and bivariate analyses. We included a random intercept for hyena ID to account for non-independence between repeated measures of corticosterone from adult hyenas (see Figure 4.3). We restricted both the maternal care and the social network data sets to include only hyenas having both an early life DNA methylation measurement and an adult fecal corticosterone measurement (Figure 4.2.), given our mediation hypothesis.

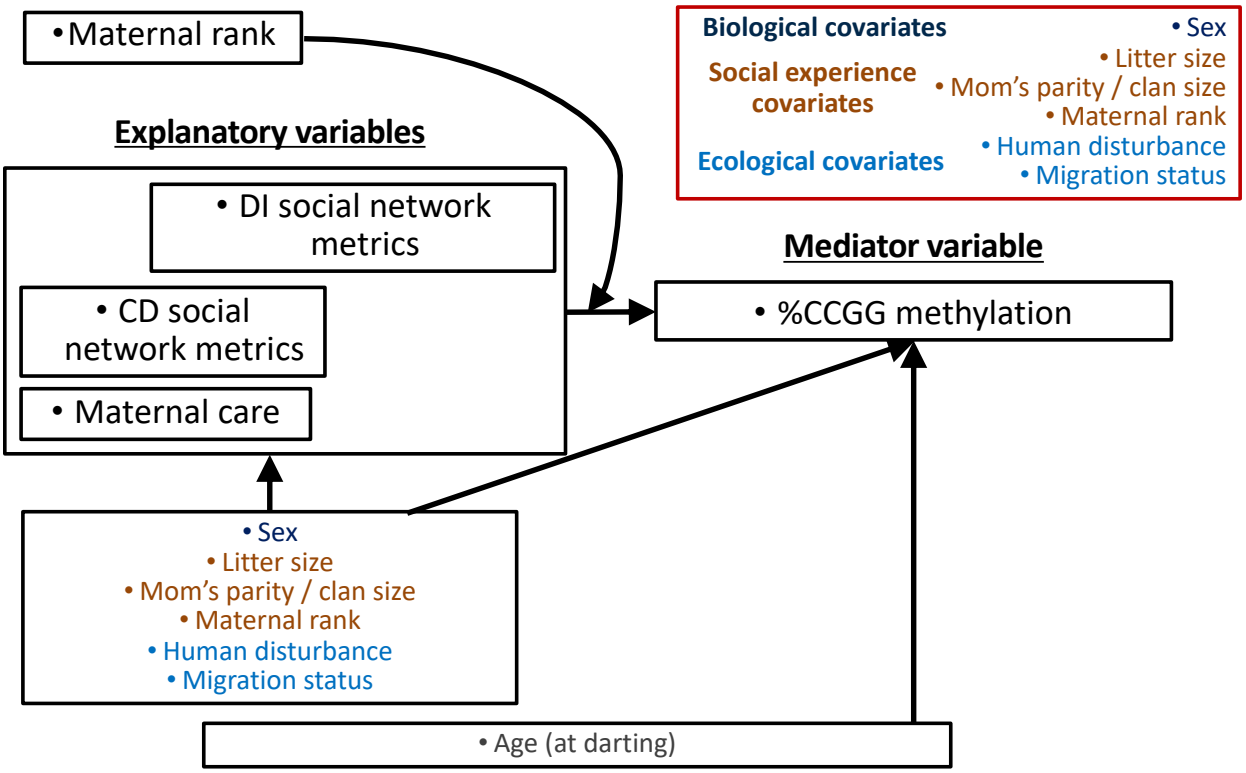


Precision covariates (gray text) are assessed when the fecal sample was collected. Edges of boxes roughly correspond to the hyenas' ages when samples were collected.

Figure 4.3. Directed Acyclic Graph (DAG) showing Step 1 of our mediation analysis: assessing associations of early life maternal care and social network metrics with adult stress phenotype. Covariates are included based on *a priori* biological knowledge and bivariate analysis (see Supplemental Table 4.4).

Step 2: Associations between maternal care behaviors or social network metrics and DNA methylation (explanatory variable → potential mediator), and between DNA methylation and adult fecal corticosterone levels (potential mediator → outcome)

We modeled the associations between each maternal care behavior and social network metric as separate explanatory variables, with %CCGG methylation as a continuous dependent variable, using linear mixed regression models. The general parameterization of these models and the hypotheses tested are depicted by the directed acyclic graph (DAG) in Figure 4.4.



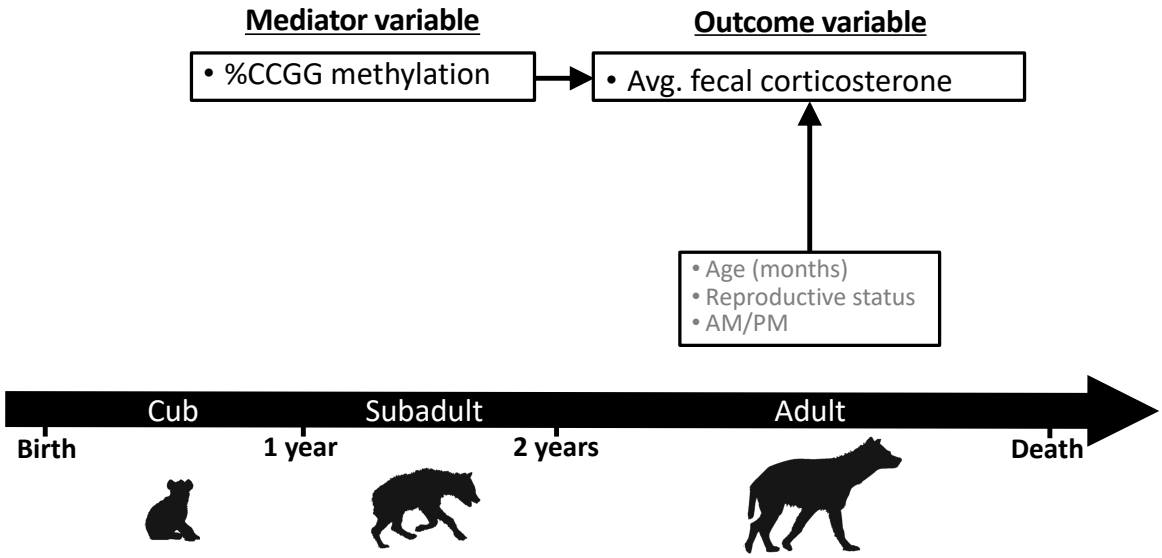
Covariates are modeled in three groups according to different contexts, which include biological covariates (dark blue), early life social experience covariates (dark green), and ecological covariates (light blue). While the temporal association between variables were maintained in our models, the timing of variables is not accurately depicted in this DAG for clarity.

Figure 4.4. Directed Acyclic Graph (DAG) showing Step 2 of our mediation analysis: assessing associations of early life maternal care and social network metrics with %CCGG methylation. Covariates are included based on previous findings from this cohort and bivariate analyses.

In each of the models where a particular maternal care behavior was the explanatory variable of interest, we controlled for three distinct categories of covariates that may influence the relationship between mother-offspring interactions and offspring DNA methylation in separate models: Model 1: biological characteristics (offspring sex); Model 2: early life social experience (number of littermates, mother's parity, and mother's social rank), and Model 3: ecological conditions (human disturbance and migration status on the date offspring were born) as potential confounding variables. We also investigated relationships between social network

metrics during the CD and DI phases of development, separately, with %CCGG methylation. We controlled for the same categories of confounding variables as described above for maternal care, with the exception that we included clan size rather than the mother's parity in the latter models. In all models, we controlled for offspring age in months as a precision covariate since age is a known determinant of DNA methylation in this and other species (Laubach, Faulk, et al., 2019). Given the importance of maternal rank to offspring DNA methylation (Laubach, Faulk, et al., 2019), and the potential for maternal rank to mitigate or exacerbate mother-offspring interactions (Engh et al., 2000; Greenberg, 2017), we tested for effect modification by maternal social rank on the relationships between each explanatory variable and DNA methylation. We included the maternal rank interaction terms in our biological covariates adjusted models, which controlled for offspring sex and age, and set the threshold for a significant interaction $p = 0.10$. None of the interactions were significant, so we did not conduct analyses stratified by maternal rank.

Next, we assessed the relationship between early life DNA methylation with adult fecal corticosterone. In these linear mixed regression models, we followed the same parameterization as described in Step 1 above, except that we replaced the maternal care/social network metrics as our explanatory variable with %CCGG methylation (Figure 4.5.).



Precision covariates (gray text) are assessed when the fecal sample was collected. Edges of boxes roughly correspond to the hyenas' ages when samples were collected.

Figure 4.5. Directed Acyclic Graph (DAG) showing step 3 of our mediation analysis: assessing associations of %CCGG methylation with adult stress phenotype. Covariates are included based on *a priori* biological knowledge and bivariate analysis (see Supplemental Table 4.4.).

Step 3: Mediation analysis

The third step of our analysis was to compare the extent to which maternal care and social network metrics during the cub and subadult life stages were associated with adult fecal corticosterone levels before and after adjustment for the potential mediator, %CCGG methylation, following Baron & Kenny (1986). If maternal care or social network metrics were associated with fecal corticosterone (Step 1) as well as %CCGG methylation, and if %CCGG methylation was associated with fecal corticosterone (Step 2), then we included %CCGG as an additional covariate in models in which each early life social variable was the explanatory variable and fecal corticosterone was the outcome. Then we compared the estimate of

association for each early life social variable with and without inclusion of the DNA methylation mediator (Baron & Kenny, 1986). In these analyses, we considered evidence for mediation by DNA methylation if inclusion of DNA methylation variable of interest attenuated the estimate for a given early life social metric by >10%.

RESULTS

Descriptive statistics

This study sample comprised four overlapping data sets based on availability of variables of interest. There were 55 hyenas with both %CCGG methylation measurements and maternal care FAS data. Mother and offspring hyena pairs spent a mean ± SD of the proportion of time they were observed together during FAS sessions in close proximity (0.852 ± 0.094), nursing (0.464 ± 0.062), and grooming (0.076 ± 0.047). Among the 86 hyenas for which we had both CD association index social network metrics and %CCGG methylation data, the mean ± SD was 55.48 ± 13.46 for degree centrality, 5.80 ± 2.09 for strength, and 7.52 ± 13.46 for betweenness centrality. During the DI stage we had social network and DNA methylation data from 81 hyenas and the mean ± SD for degree, strength, and betweenness were 61.93 ± 15.70, 4.00 ± 1.45, and 6.67 ± 5.26, respectively. We estimated a mean ± SD of fecal corticosterone of 112.16 ± 100.28 ng/g for 97 hyenas that had both a DNA methylation measurement and at least one adult fecal sample. Additional background characteristics for our FAS, social networks, and fecal corticosterone data sets can be found in Tables 4.2. - 4.4. Finally, we calculated a mean ± SD of 75.53 ± 3.03 %CCGG methylation for the 186 hyena cubs and subadults in our study population.

Maternal care behaviors (proportion of mins per behavior/mins time together)	*N*	Mean ± SD or %
Close proximity	55	0.852 ± 0.094
Nursing	55	0.464 ± 0.062
Grooming	55	0.076 ± 0.047
Biolgical confounding variable		
Sex		
Female	31	56%
Male	24	44%
Social experience confounding variables		
Number of litter mates		
Singleton	10	22%
Twins	36	78%
Mother's parity		
Primiparous	8	15%
Multiparous	46	85%
Maternal rank (year offspring born)		
Standardized maternal rank (-1 = low, 1 = high)	48	0.27 ± 0.61
Ecological confounding variables		
Human disturbance (year offspring born)		
Low	9	16.5%
Medium	26	47%
High	20	36.5%
Migration status on date of birth		
Migration absent	35	64%
Migration present	20	36%
Precision covariates and offset variable		
Offspring age		
Avg. age when %CCGG DNA measured (mons)	55	14.87 ± 4.52
FAS overlap		
Avg. amount of time mom and cub present together during FAS (mins)	55	26.14 ± 14.79

FAS = Focal Animal Survey

Table 4.2. Incident rates (proportions) of maternal care behaviors when both the mother and cub are present together during FAS sampling and background characteristics for 55 hyena cubs.

	Developmental period			
	Communal den (CD)		Den independence (DI)	
Social network metrics (based on association index)	***N***	**Mean ± SD or %**	***N***	**Mean ± SD or %**
Degree	86	55.48 ± 13.46	81	61.93 ± 15.70
Strength	86	5.80 ± 2.09	81	4.00 ± 1.45
Betweeness	86	7.52 ± 13.46	81	6.67 ± 5.26
Biolgical confounding variables				
Sex				
Female	43	50%	41	51%
Male	43	50%	40	49%
Social experience confounding variables				
Number of litter mates				
Singleton	10	12%	10	13%
Twins	71	88%	66	87%
Clan size				
Number of adult females in clan during social network measurment	86	76.24 ± 10.97	81	77.58 ± 12.01
Maternal rank (year offspring born)				
Standardized maternal rank (-1 = low, 1 = high)	86	0.22 ± 0.57	81	0.24 ± 0.56
Ecological confounding variables				
Human disturbance (year offspring born)				
Low	28	32%	26	32%
Medium	41	48%	39	48%
High	17	20%	16	20%
Migration status on date of birth				
Migration absent	60	70%	57	70%
Migration present	26	30%	24	30%
Precision covariates and offset variable				
Offspring age				
Avg. age when %CCGG DNA measured (mons)	86	13.07 ± 3.49	81	13.33 ± 3.39
Social network period length				
Avg. duration bettween first and last date of social network period (mons)	86	7.08 ± 1.79		6.95 ± 1.66

Table 4.3. Social network metrics based on association index during the communal den dependence and den independence periods and background characteristics for 81-86 hyena cubs and subadults.

Fecal Corticosterone	N_{Hyenas}	Mean ± SD or %
Concentration (ng/g)	97	112.16 ± 100.28
Potential confounding variables		
Sex		
Female	61	63%
Male	36	37%
Potential precision variables (assessed on fecal collection date)	$N_{samples}$	**Mean ± SD or %**
Age (months)	421	61.25 ± 37.87
Reproductive state		
Nulliparous	67	16%
Pregnant	50	12%
Lactating	186	44%
Other	33	8%
Male	85	20%
Time of day		
AM	166	39%
PM	255	61%
Migration status		
Migration absent	263	62%
Migration present	158	38%
Human disturbance (year offspring born)		
Low	24	6%
Medium	15	37%
High	241	57%

Table 4.4. Background characteristics for 421 fecal corticosterone samples collected from 97 adult (>24 months old) hyenas, and for which %CCGG DNA methylation also exist when hyenas were < 24 months old.

Bivariate analysis

In bivariate analyses, we assessed crude associations between potential confounding and precision covariates with each variable of interest, namely maternal care FAS behaviors, association social network metrics and fecal corticosterone. These associations have been published previously (Greenberg, 2017; Laubach, Faulk, et al., 2019; Turner et al., 2018), and we do not discuss an interpretation of the bivariate results here.

Focusing first on maternal care behaviors, we observed that offspring spent a greater proportion of time nursing (0.04, SE: 0.02, P = 0.041) from primiparous than multiparous

mothers. Offspring born in high human disturbance spent a higher proportion of time in close proximity (0.09, SE: 0.03, P = 0.008) and nursing (0.08, SE: 0.02, P < 0.001) than those born in low human disturbance. Finally, offspring born during the annual wildebeest and zebra migration spent a slightly lower proportion of their time nursing (-0.03, SE: 0.02, P = 0.094) and being groomed by their mothers (-0.02, SE: 0.01, P = 0.035) than those born when the migration was absent. The complete list of maternal care bivariate associations can be found in Supplemental Table 4.1.

We found that during both the CD and the DI periods of development, hyenas had generally higher overall connectivity as assessed by association network metrics of degree, strength and betweenness, if they were part of a larger clan (Supplemental Tables 4.2., 4.3.). During the DI period, hyenas that were born as singletons had slightly higher betweenness (3.90, SE: 2.33, P = 0.099) than hyenas from twin litters. We also observed that for both CD and DI periods, hyenas born in medium disturbance had lower degree than hyenas born in low disturbance (Supplemental Tables 4.2., 4.3.). Conversely, hyenas born in high disturbance had higher degree and strength than those born in low disturbance (Supplemental Tables 4.2., 4.3.). In DI networks only, we saw that hyenas born in medium disturbance had lower betweenness (-2.67, SE: 1.31, P = 0.045) than those born in low disturbance. Lastly, during the CD stage only, we found that being born during the migration, as compared during non-migration time periods, corresponded with lower degree (-7.41, SE: 3.08, P = 0.018) and strength (-1.98, SE: 0.44, P < 0.001).

Our analyses revealed that males have ~47% lower corticosterone (SE: 15%, P < 0.001) than females. Pregnant and lactating females both had higher fecal corticosterone levels than

did nulliparous females (Supplemental Table 4.4.). We observed approximately 0.4% higher fecal corticosterone (SE: 0.02%, P = 0.008) for each 1 month of age among our study population. Fecal samples collected in the morning had higher corticosterone concentrations than did samples collected in the evening (43%, SE: 10%, P <0.001). In this subset of 421 fecal samples from 97 individual hyenas, we saw no effect of migration status or human disturbance on fecal corticosterone levels on the date the sample was collected (Supplemental Table 4.4.).

Main analysis results

Step 1: Associations between early life social environment and adult fecal corticosterone (explanatory variable → outcome)

Hyenas that received greater amounts of maternal care as cubs had lower adult fecal corticosterone levels. Specifically, each one standard deviation (1-SD) increase in proportion of time being groomed as a cub corresponded to 32% (95% CI: 9, 45%) lower adult fecal corticosterone concentrations after controlling for the hyena's age, reproductive state, and time of day when the fecal sample was collected (Table 4.5.).

	β (95% CI) fecal corticosterone[†]			
	Unadjusted model	***N***	**Adjusted model[‡]**	***N***
Maternal Care FAS (per 1-SD)				
Close proximity	-0.01 (-0.33, 0.32)	30	-0.04 (-0.34, 0.25)	30
Nursing	-0.27 (-0.58, 0.04)	30	-0.18 (-0.50, 0.12)	30
Grooming	**-0.34 (-0.62, -0.06)**	30	**-0.38 (-0.59, -0.09)**	30
CD period (per 1-SD)				
Degree	-0.17 (-0.34, 0.00)	52	-0.13 (-0.28, 0.02)	52
Strength	-0.10 (-0.28, 0.07)	52	-0.04 (-0.19, 0.12)	52
Betweenness	0.00 (-0.26, 0.24)	52	-0.04 (-0.26, 0.17)	52
DI period (per 1-SD)				
Degree	**-0.20 (-0.36, -0.04)**	52	**-0.15 (-0.30, -0.01)**	52
Strength	**-0.19(-0.35, -0.03)**	52	-0.12 (-0.27, 0.03)	52
Betweenness	-0.11 (-0.28, 0.07)	52	-0.02(-0.18, 0.14)	52

[†]Beta estimate are fecal corticosterone concentrations (ng/g) on the natural log scale from mixed models in which hyena ID was included as a random intercept.

[‡] Models are adjusted for hyena's age (months), reproductive state/sex, and the time of day when the fecal sample was collected.

Table 4.5. Association of maternal care and early life social network metrics with adult fecal corticosterone.

Neither the proportion of time spent in close proximity or nursing were associated with adult fecal corticosterone levels.

In addition to maternal care, we also investigated the effect of early life social connections on adult stress phenotype. We found no associations between degree, strength or betweenness and fecal corticosterone among CD cubs. However, in DI hyenas, both higher degree and strength were associated with lower adult corticosterone levels: -18% (95% CI: -30%, -3%) and -17% (95% CI: -30%, -4%,), respectively (Table 4.5.). These associations were robust to adjustment for the hyena's age, reproductive state, and the time of day when the fecal sample was collected (degree [-14, 95% CI: -26, -1]; strength [-11, 95% CI: -24, 3] % difference in corticosterone concentration).

Step 2: Associations between maternal care behaviors or social network metrics and DNA methylation (explanatory variable → potential mediator), and between DNA methylation and adult fecal corticosterone levels (potential mediator → outcome)

Here, we observed multiple positive associations that were robust to various categories (biological, early life social experience, and ecological) of covariate adjustment. Starting with Model 1, which adjusted for offspring sex and age, each 1-SD greater proportion of time spent in close proximity to their mothers corresponded with 1.46% (95% CI: 0.86, 2.06) greater %CCGG methylation in cub and subadult offspring. This association was robust to adjustment for social experience covariates in Model 2, ecological covariates in Model 3, and the mutual adjustment for all covariates in Model 4 (Figure 4.6.). Similarly, more time spent nursing was also associated with higher %CCGG methylation in Models 1 and 2: 0.84% (95% CI: 0.11, 1.54) and 1.06% (95% CI: 0.07, 2.07), respectively. However, adjustment for ecological covariates in Model 3 (human disturbance and migration status) attenuated the effect of nursing time on %CCGG methylation (Figure 4.6.). There was no effect of proportion of time spent grooming on %CCGG methylation. Maternal care and maternal rank interactions were not significant.

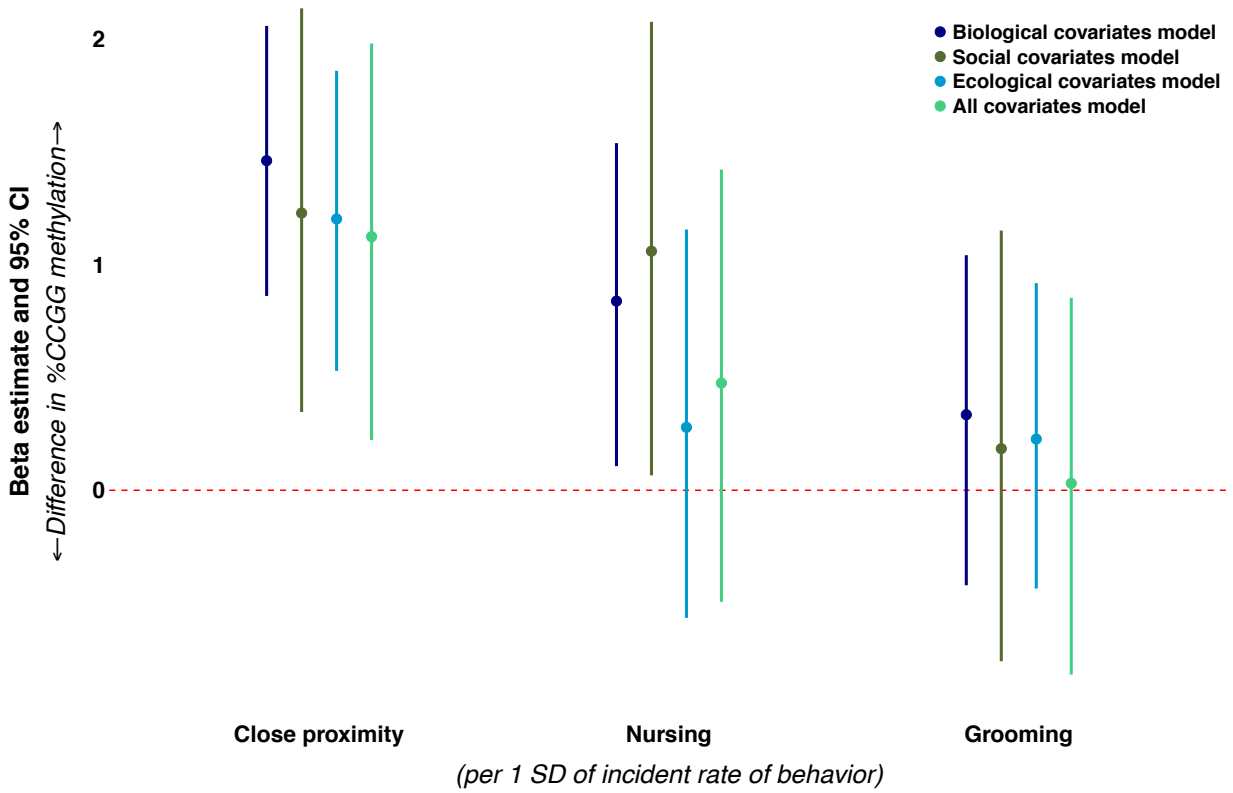


Figure 4.6. Associations of maternal care behaviors with cub and subadult offspring %CCGG methylation. All models include a random intercept for the ID of the mother hyena and are controlled for the offspring's age in months when the blood sample was collected for DNA methylation quantification. Models are further adjusted according to four contexts of covariates (biological covariates [dark blue], early life social experience covariates [dark green] and ecological covariates [light blue]). 95% CI are based on percentile parametric bootstrapping (2000 iterations).

Next, we assessed relationships between social network metrics derived during the hyenas' CD and DI phases of development with %CCGG. During the CD period, strength (0.75 [95% CI: 0.04, 1.48]) and betweenness (0.83 [95% CI: 0.06, 1.53]) were each positively associated with %CCGG methylation (Model 1) (Figure 4.7.). However, this relationship was attenuated after adjusting for early life social experience or ecological factors in Models 2 and 3 (Figure 4.7.). Degree was not associated with %CCGG methylation in any of our models during

the CD period and none of the interaction terms involving social network metrics and maternal social rank were significant.

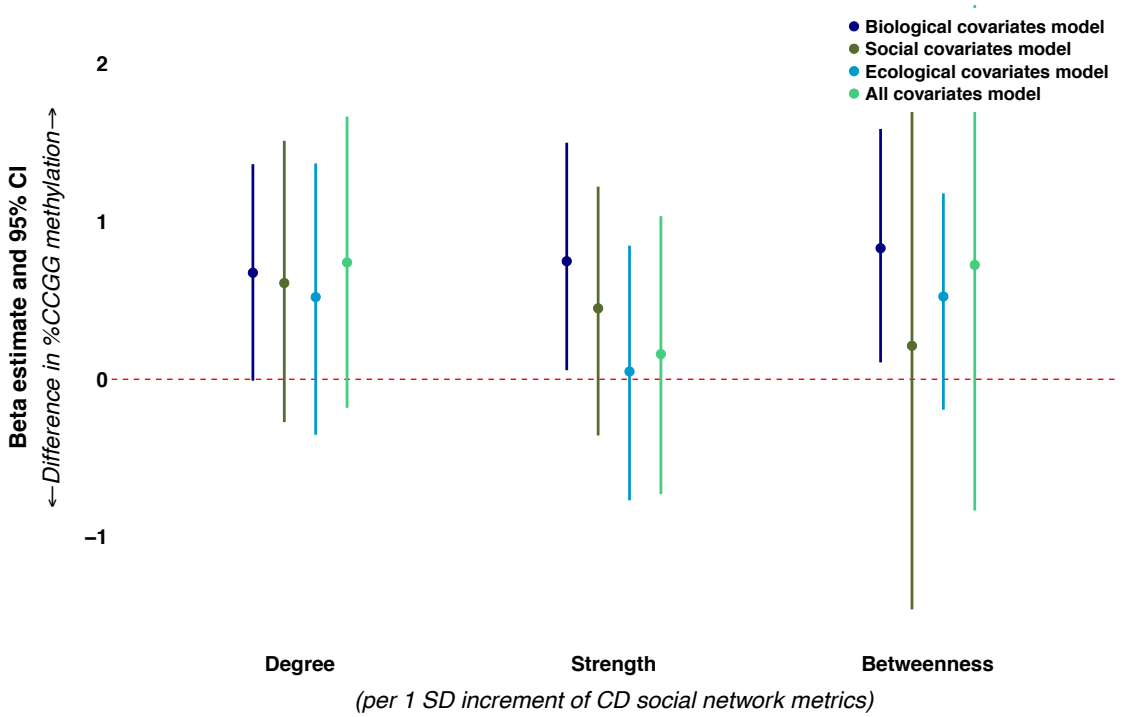


Figure 4.7. Associations of hyena CD social network metrics with cub and subadult offspring %CCGG methylation. All models include a random intercept for mother hyena ID and are controlled for the offspring's age in months when the blood sample is collected for DNA methylation quantification. Models are further adjusted according to four contexts of covariates (biological covariates [dark blue], early life social experience covariates [dark green] and ecological covariates [light blue]). 95% CI are based on percentile parametric bootstrapping (2000 iterations).

In the DI period, both degree and strength were positively associated with %CCGG. Specifically, each 1-SD increase in degree corresponded with 0.69% (95% CI: 0.00, 1.35) greater %CCGG methylation in Model 1. This association was strengthened after accounting for ecological covariates in Model 3 (1.04 [95% CI: 0.23, 1.83] %CCGG methylation) and for all covariates in Model 4 (1.19 [95% CI: 0.19, 2.17] %CCGG methylation; Figure 4.8.). Likewise, greater network strength was associated with 0.71 (95% CI: 0.04, 1.40) higher %CCGG

methylation (Model 1). This estimate was materially unchanged after accounting for ecological covariates (Model 3: 0.68 [95% CI: -0.01, 1.41] %CCGG methylation) and for all covariates simultaneously (Model 4: 0.83 [95% CI: -0.02, 1.68] %CCGG methylation), though we note that the 95% CIs were slightly wider in these models (Figure 4.8.) – likely a result of lower power with inclusion of covariates. The associations of both degree and strength with %CCGG methylation during the DI period were attenuated towards the null when we included social experience covariates in the models (Model 2). We found no association between betweenness and %CCGG methylation, and again there was no interaction between DI network metrics and maternal rank.

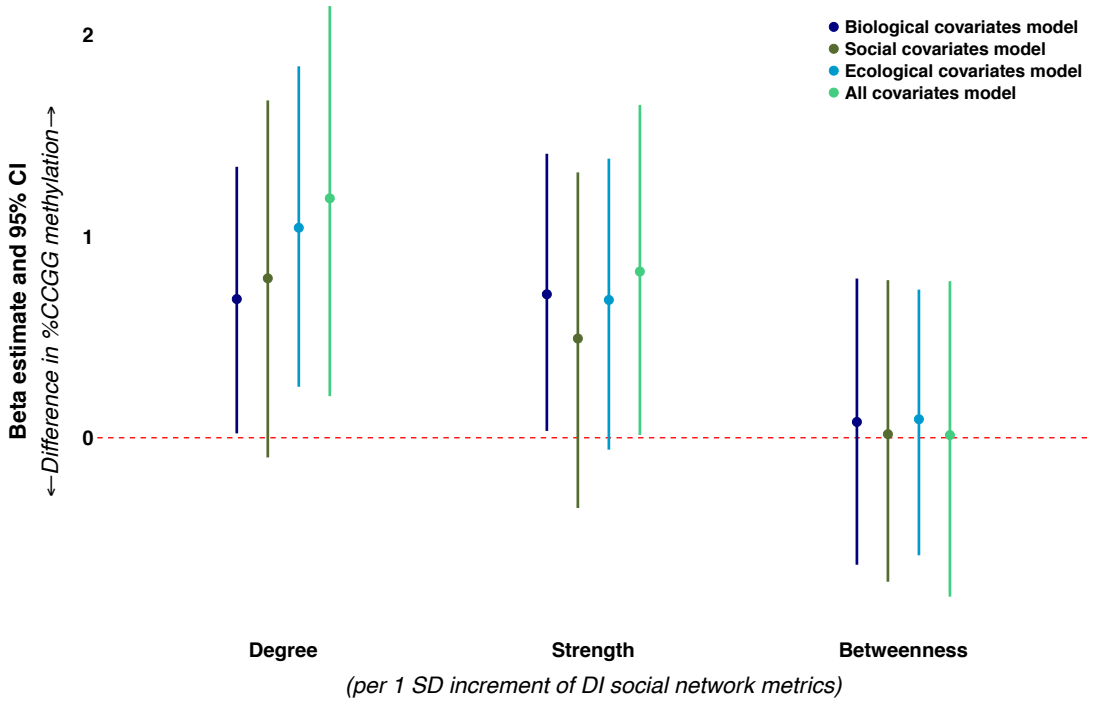


Figure 4.8. Associations of hyena DI social network metrics with cub and subadult offspring %CCGG methylation. All models include a random intercept for mother hyena ID and are controlled for the offspring's age in months when the blood sample is collected for DNA methylation quantification. Models are further adjusted according to four contexts of covariates (biological covariates [dark blue], early life social experience covariates [dark green]

Figure 4.8 (cont'd). and ecological covariates [light blue]). 95% CI are based on percentile parametric bootstrapping (2000 iterations).

As the final requirement prior to conducting formal mediation analysis, we assessed the association between %CCGG methylation and adult stress phenotype. In neither unadjusted nor adjusted models, was %CCGG methylation associated with adult fecal corticosterone (4 [95% CI: -11, 22]; 0 [95% CI: -12, 15] % difference in corticosterone concentration, respectively). Given the lack of association between our potential mediator and our adult stress outcome, we did not perform the final step of the mediation analysis.

DISCUSSION

The hypothesis that maternal care and interactions with group mates during early ontogeny influence adult physiological and behavioral phenotypes is well supported in a longstanding primate and rodent literature. More recently, primate and rodent model systems have explored molecular biomarkers, like DNA methylation, that potentially mediate the relationship between early life experience and adult stress phenotypes. However, such studies are seldom conducted with free living populations of gregarious animals, which notably are exposed to naturally occurring variation in early social experiences, and that transition through life history stages while being subject to natural selection. To address these limitations, we leveraged biological and behavioral data from a large sample of wild spotted hyenas to investigate the extent to which maternal care and social connections early in life are associated with adult stress phenotype and the potential role of DNA methylation as mediating biomarker. Broadly, we found that maternal care and interactions with group mates early in life are associated with global DNA methylation in cubs/subadults as well as adult stress phenotypes.

However, we found no evidence here that the relationship between social experience and stress phenotypes is mediated by global DNA methylation. We discuss these results in detail below following the steps of our mediation analysis.

The early life social environment and adult stress phenotype

Maternal care and adult offspring fecal corticosterone

In the first step of our analysis, maternal care received as cubs and social connectivity of subadults were both inversely associated with adult fecal corticosterone levels. Specifically, the more time a mother spent grooming a hyena during the first year of its life, the lower the offspring's fecal corticosterone concentrations in adulthood. This supports the hypothesis that higher quantity/quality of maternal care is a determinant of a well-regulated stress response later in life, which aligns with the existing literature on a range of species. For example, more licking and grooming and arched-back nursing of offspring rats perinatally is associated with lower corticosterone levels and a more sensitive stress response in offspring during adulthood (Liu et al., 1997). Cross-fostering studies in rodents have demonstrated that less maternal care, in the form of less licking and grooming and arched back nursing rates, is associated with dysregulation of genes critical for controlling the stress response (Francis et al., 1999), and ultimately elevated basal corticosterone levels (Priebe et al., 2005), suggesting a causal link between maternal care and expression of these genes. In rhesus macaques, repeated maternal separation leads to a more reactive stress response and dysregulation of daily cortisol patterns (Sánchez et al., 2005). In humans, orphaned children who remain in institutionalized care (where they receive less caregiver attention) exhibit a blunted cortisol response to a variety of

psychosocial stressors as teenagers compared to children placed into high quality foster homes or non-orphaned children (McLaughlin et al., 2015). Taken together, these results suggest that developmentally induced dysregulation of the stress response due to poor maternal care may present as inappropriate over-reaction and excessive glucocorticoid secretion, or conversely, as underreaction or a blunted glucocorticoid response to legitimate stressors. Furthermore, this body of work from controlled rodent experiments, captive non-human primates, and human studies is well aligned with our results, and suggests that greater quality or quantity of early life maternal care primes the developing mammal stress axis to function within a normal reactive scope – sufficient glucocorticoid levels above homeostatic failure and below homeostatic overload (McEwen & Wingfield, 2003; Romero, Dickens, & Cyr, 2009).

Social network metrics and adult offspring fecal corticosterone

In addition to maternal care, we found that greater social connectedness, specifically higher degree centrality, during the DI stage but not the CD stage of development corresponded with lower fecal corticosterone during adulthood. This result supports the hypothesis that social connections promote a healthy stress response. More specifically, our results imply that connections with group mates are particularly important as an animal matures, becomes independent of dens for shelter, and expands its social network beyond a limited number of associations with group mates at the communal den.

Our findings do not stand alone and corroborate what has previously been reported the literature. In two human studies of young adults, cross-sectional analyses revealed that more gregarious individuals tend to have lower salivary baseline cortisol levels (Kornienko, Clemans,

Out, & Granger, 2013, 2014; Ponzi, Zilioli, Mehta, Maslov, & Watson, 2016). Additionally, in an interview-based study of 5-year-old children, Ponzi et al. reported that the participants' perceptions of greater social network connections (i.e., higher density of friendships) was associated with lower salivary cortisol and lower salivary alpha-amylase reactivity, an indicator of reactivity to psychological stressors (Ponzi, Muehlenbein, Geary, & Flinn, 2016). Such findings further support the notion that the link between social bonds and stress phenotypes begins early in life. However, given the lack of longitudinal assessment of social network metrics and glucocorticoid levels in these human studies, little can be said about the extent to which social connections influence the stress phenotype or vice versa.

Moving outside of the human literature, a study on wild yellow-bellied marmots found no effect of social network metrics on fecal glucocorticoid levels measured in a mixed age, mixed sex population (Wey & Blumstein, 2012). Considering previous human and animal studies, our findings are notable given that we observed an association between social network metrics and glucocorticoid levels in our wild hyenas. The present study improves upon the limitations of a cross-sectional study design as we were able to examine prospective associations during two critical windows of development between early-life social network metrics and the stress phenotype measured during adulthood in hyenas. Explicitly considering the timer order between the independent and dependent variables in our models (Hill, 1965) reduces the possibility of reverse confounding bias and takes an additional step towards disentangling the potential causal relationship between the early life social connections and later life stress phenotype.

The early life social environment and DNA methylation

Maternal care and global DNA methylation

In the second step of our analysis, we found that maternal care received during the CD life stage, and social connectivity during the subadult DI life stage, were both associated with higher global DNA methylation later in life (i.e. primarily in subadults). Specifically, offspring that spent more time in close proximity to and nursing from their mothers during the first year of life had higher global DNA methylation later in life. Focusing on proximity, we found that, even after adjustment for various categories of confounding variables, including biological, social and ecological covariates assessed at birth, the positive effect of time spent in close proximity to the mother on global DNA methylation remained robust. Similarly, greater time spent nursing corresponded to higher global DNA methylation, even after covariate adjustment. However, the effect of nursing on offspring global DNA methylation was attenuated when we included early life ecological variables in the model, indicating that the ecological context may be a key confounder to the relationship between time spent nursing and global DNA methylation. This makes sense in light of previous findings of differences in maternal care behavior in relation to human disturbance and prey availability in our hyenas (Greenberg, 2017). Nevertheless, our results support the hypothesis that greater maternal care is associated with an epigenetic biomarker that serves as an indicator of genomic stability and, purportedly, better overall health (Eden et al., 2003; Feinberg & Vogelstein, 1983; Gaudet et al., 2003; Tuck-Muller et al., 2000).

The above-mentioned results align with our predictions, which were based on the primate and rodent literature. In rodents, more maternal licking and grooming is associated with lower DNA methylation of CpG sites in the promoter region of the glucocorticoid receptor gene, which is involved in the regulation of the stress response (Szyf et al., 2005; Weaver et al., 2004, 2007). In captive rhesus macaques, maternal deprivation, in which a young monkey's mother is replaced with an inanimate surrogate, is related to marked differences in DNA methylation of genomic regions involved in the stress response (e.g., immune function and neurodevelopment pathways) in adult offspring (Massart et al., 2014; Provencal et al., 2012).

Social network metrics and global DNA methylation

In addition to maternal care, social connections during the DI but not CD stage of development were positively associated with global DNA methylation. Degree and strength of social connections, both of which reflect the number of group mates with which a hyena associates, corresponded with higher global DNA methylation even after adjustment for biological and ecological covariates. Controlling for early life social covariates slightly attenuated the effect of social connections and shifted the confidence intervals across the null. Confounding by early life social covariates of the relationship between social network metrics and global DNA methylation is not unexpected given that maternal rank, parity, and number of littermates are known to affect the development of young hyena's social network (Turner et al., 2018) and are also determinants of global DNA methylation (Laubach, Faulk, et al., 2019). Overall our results suggest that greater social connectivity during the DI period is positively associated with an epigenetic biomarker of health.

In both human and non-human primates, a number of studies have identified social rank and socioeconomic status as key determinants of DNA methylation. Social rank in captive rhesus macaques was associated with differences in genome-wide patterns of DNA methylation at birth (Massart et al., 2017) and in adulthood (Tung et al., 2012b). Similarly, in humans, socioeconomic status is related to patterns of differential methylation measured across the life span (Laubach, Perng, et al., 2019). Although there is strong evidence that patterns of DNA methylation vary with social status, both dominance rank and socioeconomic status are broad social constructs that encompass, not only social positions and interactions among individuals within a group, but also access to resources. Our study is noteworthy because it is one of the first to investigate the relationship between social network metrics of connectedness, specifically, with patterns of DNA methylation in either the biomedical sciences or ecology and evolutionary biology.

DNA methylation and adult fecal corticosterone

Global DNA methylation assessed in cub and subadult hyenas was not associated with adult fecal corticosterone. Therefore, in this study sample, global DNA methylation does not appear to mediate the relationship between early life experience and adult stress phenotype. Alternative explanations for this lack of an association in the final leg of our mediation analysis and general limitations are discussed below.

Limitations and future directions

One explanation for the lack of evidence for direct mediation by global DNA methylation is that this composite measure of total CCGG% methylation, averaged over the entire genome, is too broad to capture the effect of differential methylation of specific gene regulatory pathways involved in the stress response. An alternative approach could involve the use of Reduced Representation Bisulfite Sequencing (Meissner et al., 2005), a high throughput sequencing technology that assays single nucleotide DNA methylation resolution at a genome wide scale. In fact, a number of the human and laboratory animal studies referenced above, which have found associations between early life social experiences and DNA methylation, use high throughput sequencing techniques (Laubach, Perng, et al., 2019; Tung et al., 2012b). We, too, are employing this method in a pilot sample. However, the tradeoff between a large sample size using the relatively inexpensive LUMA assay compared to the much more costly next generation sequencing approach that reduces sample size (and thus, statistical power to detect associations) should be carefully considered.

Second, fecal corticosterone concentrations may not capture the nuances of stress phenotype. Despite widespread use of fecal corticosterone as an average baseline stress hormone measurement (Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011), these estimates can vary substantially based on unmeasured environmental factors (e.g., we do not know whether or not, before defecating a hyena was chased by a lion) and individual level differences of the animal from which the sample is collected (Goymann, 2012), thus making relative comparisons challenging. In this way average levels of glucocorticoids derived from fecal samples fail to provide accurate information regarding the functioning of an individual's acute

stress response. Future studies might consider other measures of adult stress phenotypes or health outcomes, such as glucocorticoid hormone recovery from a standardized stressor or hormonal challenge (e.g. ACTH [Adrenocorticotropic hormone]), stress related behaviors (e.g. boldness and neophobia), telomere length, or immune function. Ultimately, future studies should also thoroughly investigate each of the links along a pathway from early social environment, through molecular biomarkers that influence adult phenotype, and culminate in an effect on fitness.

Finally, our assessment of DNA methylation was limited to whole blood, and without information on cellular composition. However, our interpretation of global DNA methylation as a biomarker does not preclude differences in DNA methylation that are potentially due to changes in whole blood cellular composition; instead, changes in cellular composition that correspond with variation in global DNA methylation estimates from a heterogenous cell population are part of the total effect of the early life social environment on a global DNA methylation biomarker.

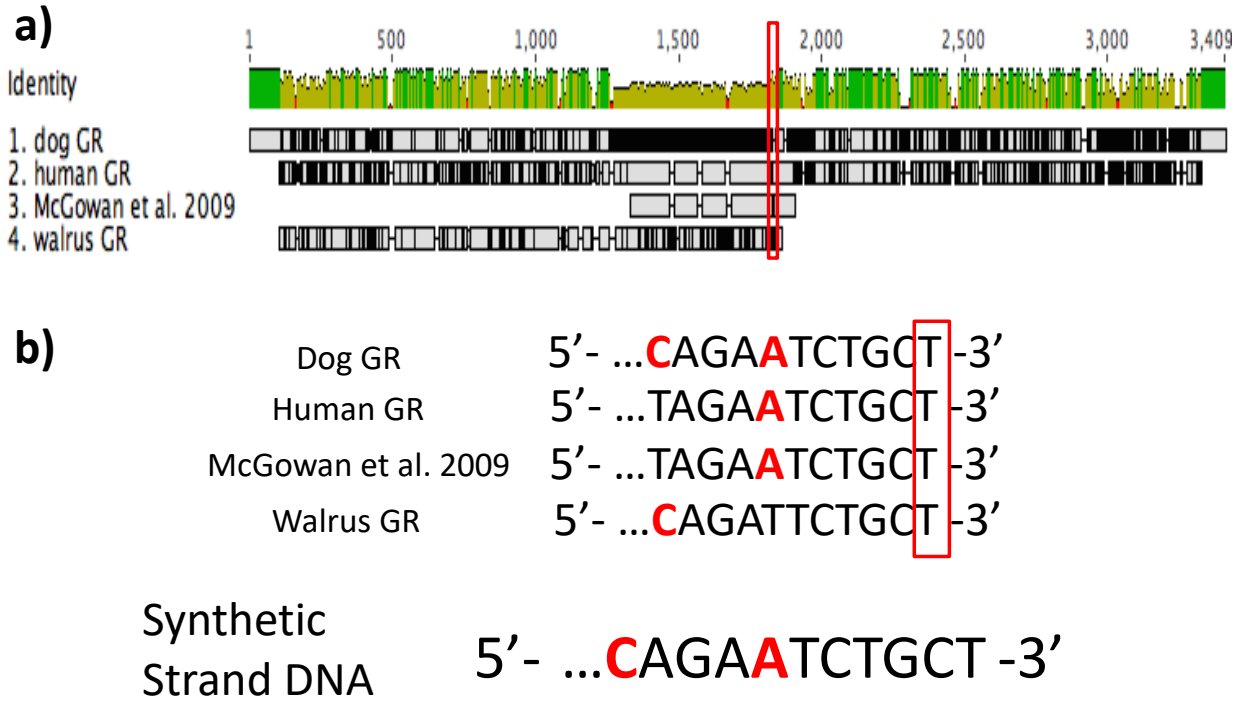
CONCLUSIONS

For the first time in a wild population, we found robust evidence that maternal care and social connections with group mates early in life are associated both with DNA methylation early in life and fecal stress hormone concentrations in adulthood. More specifically, we found that greater maternal care in the first year of life and greater social connectedness in den independent offspring correspond to higher global DNA methylation and lower baseline glucocorticoid levels later in life. These results suggest that multiple aspects of social

experience contribute to the developmental plasticity of stress phenotypes, and that the timing of these experiences matters. While we did not observe any associations between global DNA methylation and adult fecal stress hormone levels, which indicates that global DNA methylation does not appear to mediate the relationship of interest, our work with hyenas joins a growing body of evidence from rodents and primates highlighting the critical effects of early life experience on molecular biomarkers and developing stress phenotypes.

SUPPLEMENTAL INFORMATION

We used a target gene approach to measure DNA methylation of CpGs within the putative promoter region of the spotted hyena glucocorticoid receptor (GR) gene. To do this we *de novo* sequenced the GR promoter region of DNA in hyenas. Given that there was no publicly available hyena genome, we first made a 'synthetic strand of DNA,' which was intended to represent our best guess at the nucleotide sequence for hyenas and which was based on the consensus base pairs from the alignment of multiple species' GR promoter sequences (Supplemental Figure 4.1).

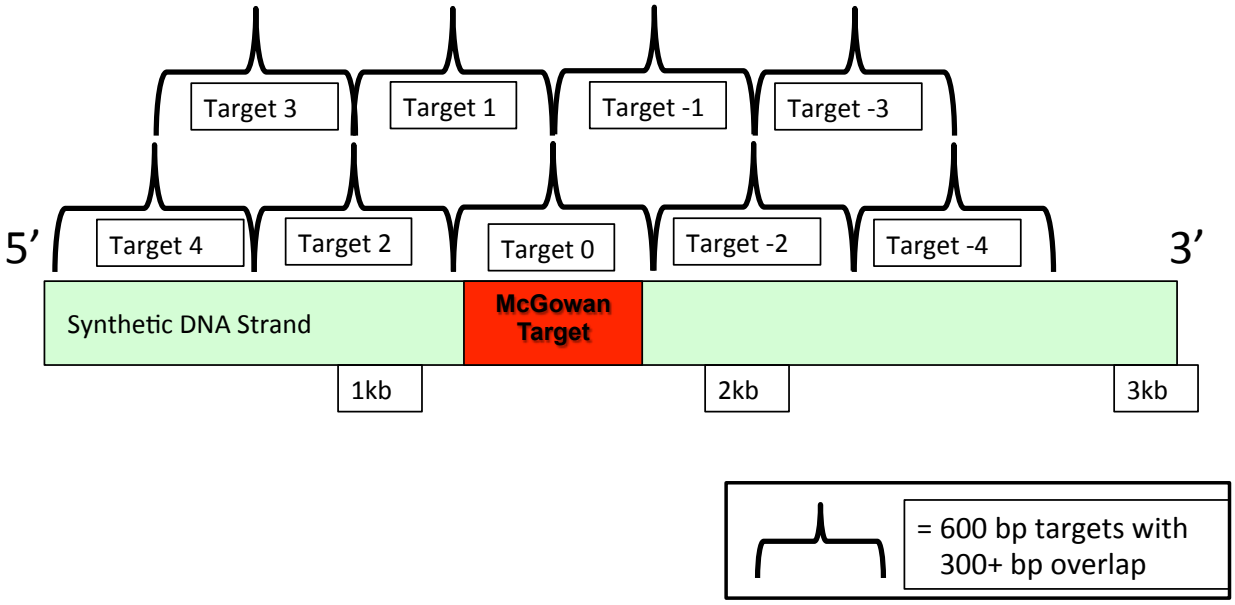


Supplemental Figure 4.1. (a) A multiple species alignment of the promoter region of the Glucocorticoid Receptor, where consensus nucleotide identity is represented by the green bar at the top. (b) A zoomed in view of the nucleotides from each species in the alignment and the 'synthetic strand of DNA' at the bottom. Red boxes represent the sliding window used to assess nucleotide consensus at each position and red nucleotides indicate a mismatch between one or more species.

More specifically, our multiple species alignment contained human (including from McGowan *et al.* 2009), domestic dog, and walrus GR promoter DNA. Then moving across the alignment, we selected the nucleotide for our 'synthetic strand of DNA' at each position based on the greatest nucleotide similarity across species or based on the nucleotide from species most closely related to hyenas when there was no clear consensus.

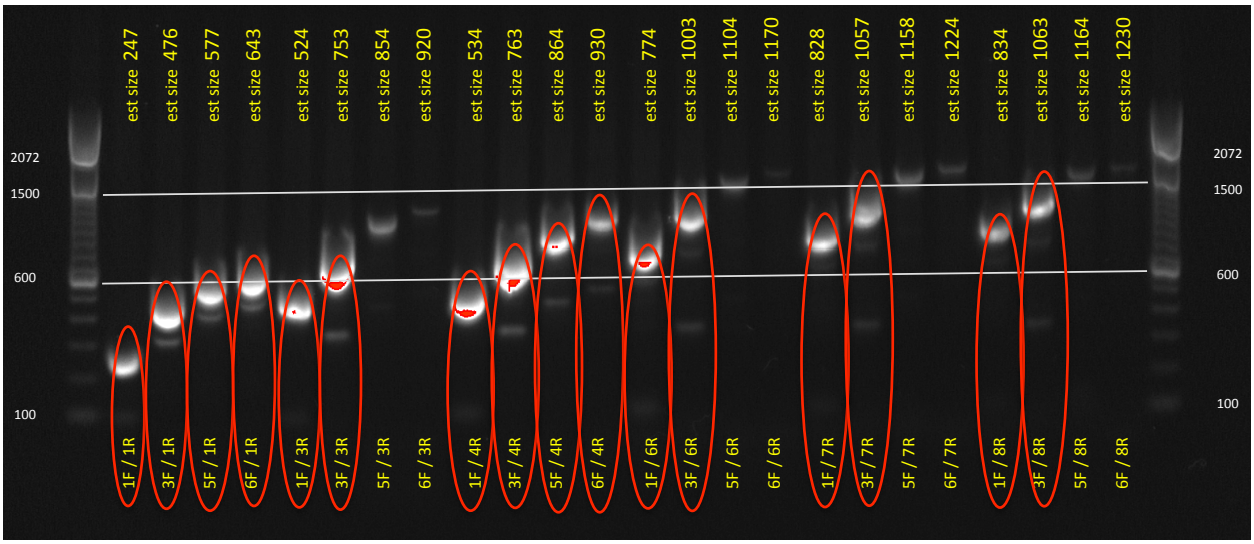
Next, using our synthetic strand of DNA, we designed a tiling array of PCR primers with overlapping amplification products that targeted the GR promoter region, including parts of this

promoter that had been shown to be differentially methylated in human and rodent studies (c.f. [McGowan et al., 2009], Supplemental Figure 4.2).



Supplemental Figure 4.2. Tiling array of PCR primers and targets aimed at generating overlapping DNA sequence from spotted hyenas that aligned with GR promoter region and included sequence of interest identified in human DNA methylation studies (e.g. McGowan target).

We size selected spotted hyena DNA PCR products from gels based on the estimated amplicon lengths from our 'synthetic strand of DNA,' and Sanger sequenced the size matched products (Supplemental Figure 4.3). In Sanger sequencing, we used genomic DNA extracted from whole blood samples taken from three individual hyenas.



Supplemental Figure 4.3. A gel image showing the PCR products from our tiling array. Size ladders are shown in the first and last wells. Primer sets, identified on the bottom of the gel, and their respective DNA bands are circled in red if the PCR product matched the estimated size. Estimated sizes were based on the 'synthetic strand of DNA' and are listed across the top of the gel image.

We then aligned the overlapping hyena DNA reads from the Sanger sequencing and trimmed the hyena DNA to match published human GR DNA sequence (McGowan et al., 2009; Oberlander et al., 2008; Perroud et al., 2011), which resulted in the assembly of the putative GR promoter region for spotted hyenas (Supplemental Figure 4.4).

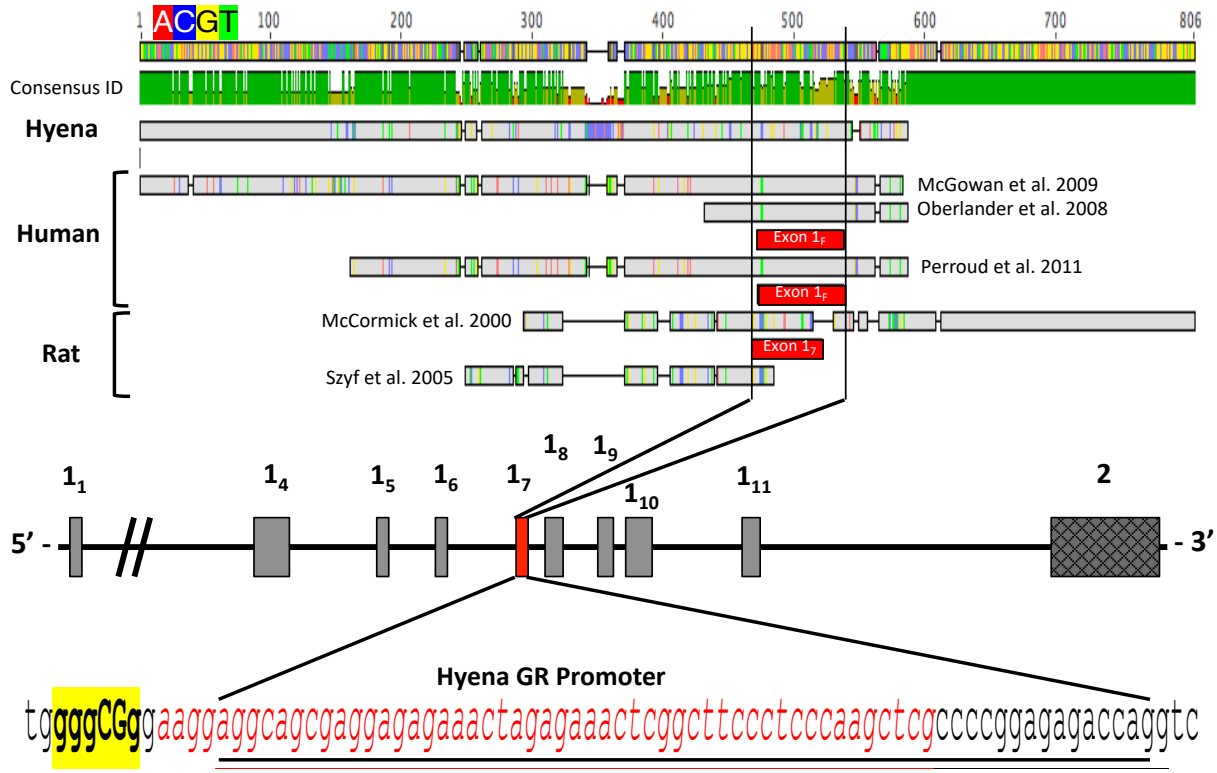
Putative spotted hyena GR Promoter

5' - gggttctgctttgcaacttctctcccggtgggagagcgcgacggcggcgg
cggctgcagacgggg**cCGccc**agacgatgcggcggtgggggacctgccgg
cacgcgactacccccgagtcgagagtatgtacgcaccgacccccctcctct
ctcctcccctccctcagcctccccagagggcgtgtctggctgtccggccc
cgagcgaggccgagacgctgcggcaccgtttccttgcaaccctcgtagcc
ccgtgctaagtgacacagttcgcgcaactccgctccgaggcggcggcgcg
gaccactcccgcggccccgctgcgggccgcgccccggcctccccccaccc
cccacc**cCGccc**cagacagcccgctgtcacccgcaggggcgctgacggcg
ccggtcgccgagggactgggccagctccggagtgggtcgggagtcgcgcc
gctgggctg**gggCGg**g*aaggaggcagcgaggagagaaactagagaaactc*
*ggcttccctcccaagctcg*ccccggagagaccaggtcggcctccagccgc
cacctctccctttcccggagggtgggggg **- 3'**

Supplemental Figure 4.4. Spotted hyena putative GR promoter sequence, which has been trimmed to match the aligned sequences published in Oberlander et al. 2008, McGowan et al. 2009, and Perroud et al. 2011. Underlined is the inferred consensus region (based on

Supplemental Figure 4.4 (cont'd). alignment) for human exon 1_F, and red, italicized is the inferred consensus region (based on alignment) for rat exon 1_7. GC boxes, the putative binding site for transcription factor NGFI-A, are **bold** and contained CpG sites capitalized. The yellow highlighted GC box is potential NGFI-A transcription binding site homologous to the region in rats that was shown to be differentially methylated with respect to maternal licking and grooming (c.f. Weaver et al. 2004).

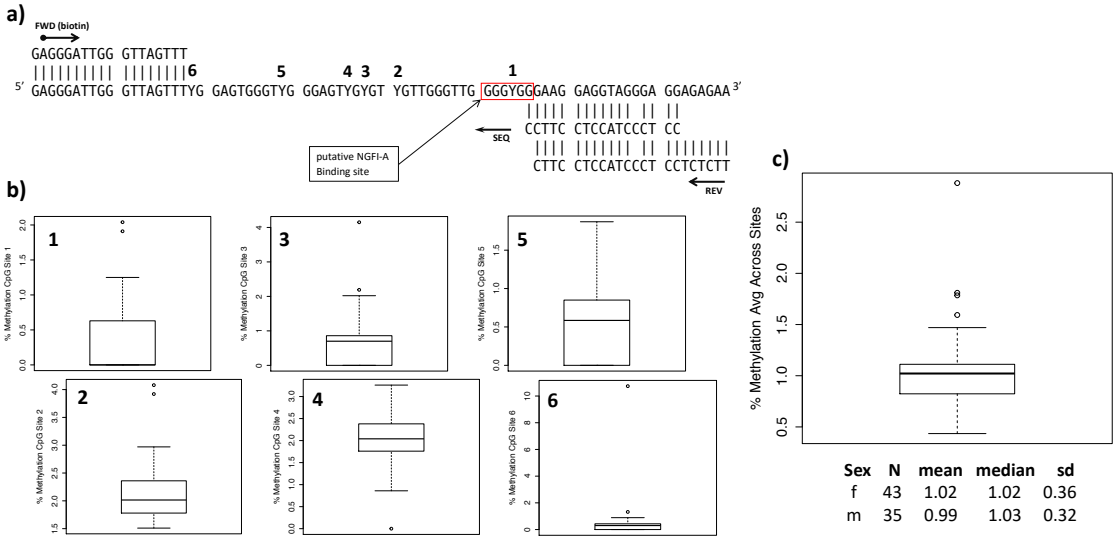
After sequencing the putative GR promoter in hyenas, we aligned our trimmed sequence with human and rat DNA sequences that were reported in the literature to be differentially methylated with respect to early life adversity and maternal care, respectively (Mccormick et al., 2000; McGowan et al., 2009; Oberlander et al., 2008; Perroud et al., 2011; Szyf et al., 2005; Weaver et al., 2004) and as shown in Supplemental Figure 4.5.



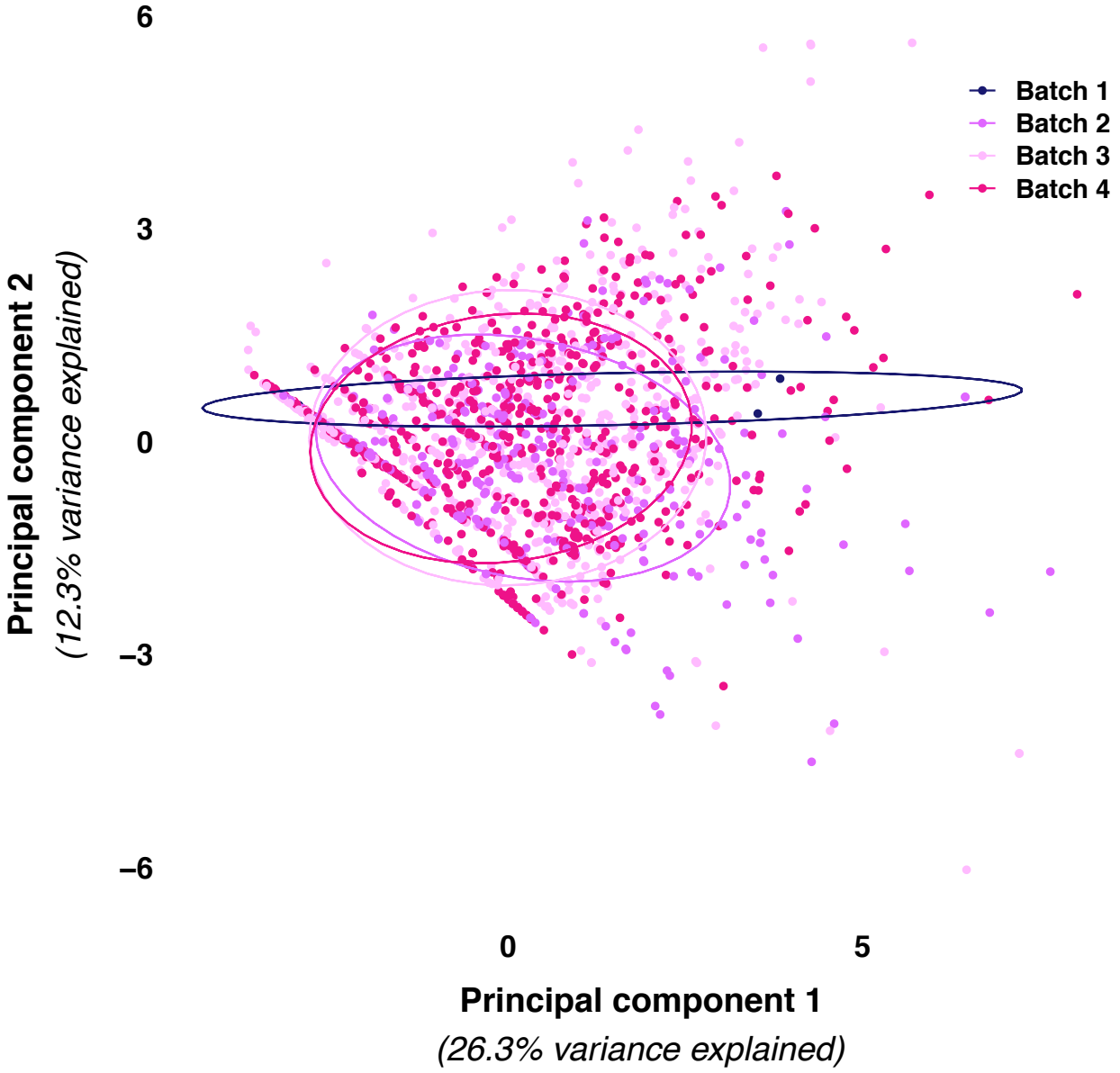
Supplemental Figure 4.5. Cross species comparison (spotted hyenas, humans and rats) of the GR promoter region that is the target of gene-specific DNA methylation studies. Base pair consensus from amplicons reported in multiple published studies as well as our spotted hyena amplicon are shown. Focusing on Exon $1_F/1_7$ and the base pair resolution of the hyena GR promoter, we show hyena DNA sequence that corresponds with human exon 1_F, underlined,

Supplemental Figure 4.5 (cont'd). and with rat exon 1_7, red and italicized. Also identified in yellow highlighted and bold text is the putative transcription factor binding site for NGFI-A.

Focusing on an approximately 50 base pair region which overlapped with the differentially methylated regions in human and rat studies (Supplemental Figure 4.5), including the putative transcription factor binding site for NGFI-A, we quantified CpG methylation using pyrosequencing on a Qiagen Pyromark® Q96 MD. More specifically, we bisulfite treated genomic DNA extracted from whole blood, and then measured % DNA methylation in 96 hyena samples (Supplemental Figure 4.6) at 6 CpG sites. We found near 0% DNA methylation and low variation at all 6 CpG sites, so we did not conduct further analyses of GR promoter DNA methylation in spotted hyenas (Supplemental Figure 4.6).



Supplemental Figure 4.6. Quantitative measurement of DNA methylation at 6 CpG sites in the hyena putative GR promoter in a DNA sequence that was targeted in previous rat and human studies. a) Target DNA sequence with potentially methylated cytosine bases identified with a 'Y' and numbered according to pyrosequencing order. Putative transcription factor binding site is identified in the red box and forward, reverse and sequencing primers are shown on the DNA strand. b) Boxplots showing site-specific CpG percent methylation. c) Boxplot and descriptive statistics showing percent methylation values averaged across all 6 CpG sites from 78 hyenas (note some samples did not pass the pyrosequencing quality standards, so the total samples size dropped from 96 hyenas).



Supplemental Figure 4.7. Principal Components Analysis (PCA) plot of focal animal survey (FAS) batches (data collection groupings) for maternal care behaviors. Ellipses represent 68% probability.

	β (± SE)[†]					
	Close proximity	***P***	**Nursing**	***P***	**Grooming**	***P***
Biological confounding variables						
Sex						
Female	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Male	-0.03 (± 0.03)	0.252	-0.01 (± 0.02)	0.753	-0.01 (± 0.01)	0.363
Social experience confounding variables						
Number of litter mates						
Singleton	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Twins	-0.05 (± 0.03)	0.189	0.02 (± 0.02)	0.458	-0.02 (± 0.01)	0.115
Mother's parity						
Primiparous	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Multiparous	-0.03 (± 0.04)	0.335	**-0.04 (± 0.02)**	**0.041**	-0.01 (± 0.02)	0.466
Ecological confounding variables						
Human disturbance (year offspring born)						
Low	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Medium	-0.01 (± 0.03)	0.860	-0.001 (± 0.02)	0.955	-0.02 (± 0.02)	0.267
High	**0.09 (± 0.03)**	**0.008**	**0.08 (± 0.02)**	**<0.001**	0.01 (± 0.02)	0.614
Migration status on date of birth						
Migration absent	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Migration present	-0.03 (± 0.03)	0.257	*-0.03 (± 0.02)*	*0.094*	**-0.02 (± 0.01)**	**0.035**

[†]Estimates represent differences in incident rates or proportions of time spent engaged in a particular behavior in relation to the total time the mother-offspring pair were observed together (n =

Supplemental Table 4.1. Bivariate associations of biological, early social experience, and ecological covariates with maternal care behavior BLUPs.

	β (± SE)[†]					
	Degree[†]	***P***	**Strength**	***P***	**Betweeness**	***P***
Biological confounding variables						
Sex						
Female	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Male	2.16 (± 2.91)	0.460	0.63 (± 0.45)	0.167	3.53 (± 2.90)	0.226
Social experience confounding variables						
Number of litter mates						
Singleton	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Twins	-3.78 (± 4.49)	0.403	0.27 (± 0.69)	0.695	*-3.90 (± 2.33)*	*0.099*
Clan size						
Number of hyenas in clan	**0.51 (± 0.12)**	**0.001**	0.03 (± 0.02)	0.210	*0.24 (± 0.13)*	*0.066*
Ecological confounding variables						
Human disturbance (year offspring born)						
Low	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Medium	**-9.44 (± 2.72)**	**0.001**	0.05 (± 0.48)	0.912	4.21 (± 3.29)	0.205
High	**10.73 (± 3.42)**	**0.002**	**2.14 (± 0.60)**	**0.001**	-0.69 (± 4.13)	0.869
Migration status on date of birth						
Migration absent	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Migration present	**-7.41 (± 3.08)**	**0.018**	**-1.98 (± 0.44)**	**<0.001**	-1.13 (± 3.18)	0.722

[†]Estimates are based on association index networks from 86 communal den dependent hyeans.

Supplemental Table 4.2. Bivariate associations of biological, early social experience, and ecological covariates with communal den (CD) social network metrics.

	β (± SE)[†]					
	Degree	***P***	**Strength**	***P***	**Betweeness**	***P***
Biological confounding variables						
Sex						
Female	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Male	-1.73 (± 3.51)	0.623	0.42 (± 0.32)	0.193	-0.87 (± 1.17)	0.461
Social experience confounding variables						
Number of litter mates						
Singleton	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Twins	4.11 (± 5.30)	0.441	0.61 (± 0.48)	0.205	-2.17 (± 1.76)	0.221
Clan size						
Number of hyenas in clan	**0.75 (± 0.12)**	**<0.001**	**0.03 (± 0.01)**	**0.012**	**0.16 (± 0.05)**	**0.001**
Ecological confounding variables						
Human disturbance (year offspring born)						
Low	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Medium	**-13.35 (± 3.05)**	**<0.001**	-0.44 (± 0.33)	0.183	**-2.67(± 1.31)**	**0.045**
High	**12.88 (± 3.83)**	**0.001**	**1.28 (± 0.41)**	**0.003**	-0.56 (± 1.64)	0.734
Migration status on date of birth						
Migration absent	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Migration present	-0.01 (± 3.84)	0.997	0.33 (± 0.35)	0.353	-1.69 (± 1.27)	0.190

[†]Estimates are based on association index networks from 81 communal den independent hyeans.

Supplemental Table 4.3. Bivariate associations of biological, early social experience, and ecological covariates with den independent (DI) social network metrics.

	β (± SE)[†]	
	Cort	***P***
Sex		
Female	0.00 (Reference)	
Male	**-0.64 (± 0.15)**	**<0.001**
Precision covariates (assessed on fecal collection date)		
Age (months)	**0.004 (± 0.002)**	**0.008**
Reproductive state		
Nulliparous	0.00 (Reference)	
Pregnant	**1.06(± 0.19)**	**0.000**
Lactating	**0.57 (± 0.15)**	**0.000**
Other	**0.63 (± 0.22)**	**0.004**
Male	-0.15 (± 0.18)	0.411
Time of day		
AM	0.00 (Reference)	
PM	**-0.57 (± 0.10)**	**<0.001**
Migration status		
Migration absent	0.00 (Reference)	
Migration present	0.08 (± 0.10)	0.419
Human disturbance		
Low	0.00 (Reference)	
Medium	0.34 (± 0.28)	0.224
High	0.20 (± 0.28)	0.483

[†]Beta estimate are differences in each categorical variable from the reference group in fecal corticosterone concentrations (ng/g) on the natural log scale from mixed models in which hyena ID was included as a random intercept. Models include 421 fecal corticosterone samples collected from 97 adult (>24 months old) hyenas, and for which %CCGG DNA methylation data also exist

Supplemental Table 4.4. Bivariate associations of sample collection variables and adult fecal corticosterone.

CHAPTER 5

AN EXPLORATORY ANALYSIS OF FUNCTIONAL GENOMIC REGIONS INVOLVED IN THE EARLY LIFE SOCIAL ENVIRONMENT AND ADULT STRESS PHENOTYPE: A GENOME-WIDE ASSOCIATION STUDY USING ENHANCED REDUCED REPRESENTATION BISULFITE SEQUENCING (ERRBS) DATA

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INTRODUCTION

The social status into which an animal is born can have lasting influence on its physiological and behavioral stress response (Creel, Dantzer, Goymann, & Rubenstein, 2013; McEwen & Seeman, 1999; Sapolsky, 2004). For example, higher maternal dominance rank is associated with lower baseline glucocorticoid levels in subadult male yellow baboons (Onyango, Gesquiere, Wango, Alberts, & Altmann, 2008). This pattern of low social status early in life and an adverse stress phenotype later in life extends to humans as well. Adults who experienced low socioeconomic status as children exhibit lower expression of genes involved in control of the hypothalamic pituitary adrenal (HPA) axis and elevated daily cortisol levels independent of their current social status (Miller et al., 2009), suggesting a unique “programming” effect of early social exposures on future stress phenotype.

In addition to social status, another widely studied aspect of the early life social environment is maternal care. From the crude maternal separation studies in non-human primates wherein infant primates were reared with surrogate wire mothers or periodically removed from their mothers (Harlow et al., 1965; Harlow & Zimmermann, 1958; Sánchez et al.,

2005), to variation in maternal licking and grooming in laboratory rodents (Caldji, Diorio, & Meaney, 2000; Caldji et al., 1998; Francis et al., 1999; Liu et al., 1997; Priebe et al., 2005), there is compelling evidence that the quality and quantity of maternal care plays an important role in the development of stress-related behaviors and physiology in offspring. Yet, one question that remains is *how* do aspects of the early life social environment manifest in differences in later life stress phenotypes?

While the above inquiry has historically been approached using conventional methods for assessing DNA methylation of single candidate genes (e.g., NRC31 (Weaver et al., 2004)) or global measures of DNA methylation (e.g., repetitive elements (Subramanyam et al., 2013; Tehranifar et al., 2013), the recent advent of high-throughput technologies has enabled researchers to identify novel molecular biomarkers and functional gene regulatory pathways at the genome-wide level. For example, recent work in non-human primates (Massart et al., 2017) and humans (Borghol et al., 2012) using genome-wide DNA methylation arrays demonstrated a distinct effect of maternal social status and socioeconomic status on genome wide patterns of DNA methylation, particularly in genomic regions involved in the stress response and immune function (Bush et al., 2018a; Needham et al., 2015; Stringhini et al., 2015). Although these studies champion the utility of high-dimensional epigenomics data as a means to the holy grail of identifying specific mechanisms that link early life social status to later life stress phenotypes, studies are missing that explicitly identify DNA methylation patterns associated with both the early social environment *and* future stress phenotype in the same population.

In previous chapters, we showed that maternal rank during the year in which a hyena is born (Laubach, Faulk, et al., 2019) (Chpt. 3) and maternal care (Chpt. 4) are each associated

with global DNA methylation in wild spotted hyenas. We also show that greater maternal care is associated with lower adult fecal corticosterone levels, but that the relationship between the early life social environment metrics and adult stress phenotype was not mediated by global DNA methylation (Chpt. 4), a finding that suggests the existence of alternative molecular pathways, possibly including other DNA methylation pathways, through which these relationships might operate. Therefore, in this final chapter, we leverage genome-wide DNA methylation data in a pilot sample of 30 hyenas to identify differentially methylated CpG cites that are associated with both adult stress phenotype and the early social environment via a two-step process. First, we conducted an epigenome wide association study (EWAS) in which subadult and adult fecal corticosterone levels are used to predict differentially methylated CpG sites in hyenas. Second, we investigated associations between maternal social rank and maternal care variables with offspring DNA methylation among sites identified in the EWAS. Differentially methylated sites (DMSs) that were 'predicted' by both fecal corticosterone and early life social environment factors are identified as potential biomarkers of gene regulatory pathways important to the etiology of a socially induced adverse stress phenotype.

METHODS

Study population

Our study population comprised 30 spotted hyenas from the Mara Hyena Project, a long-term field study in the Masai Mara National Reserve, Kenya. We selected animals for this pilot study based on strict selection criteria due to budgetary restrictions. First, all animals in the analytical sample were females from a single social group (the Talek clan) that were born

between 2011-2014 to reduce variability in background characteristics, with the exceptions of one hyena born in 2010 and another born in 2007. Next, because we were interested in DNA methylation as a potential molecular marker linking the early social environment (maternal rank and maternal care) and future stress phenotype (adult fecal corticosterone concentrations), we further restricted the data set to hyenas for which we had at least 100 µL of 50 ng/µL of DNA extracted from whole blood during the subadult and adult life stages. Of the 30 hyenas with adequate DNA concentrations, 25 also had information on fecal corticosterone concentrations during the subadult or adult life stages; these individuals comprised the study sample for the EWAS. We restricted the fecal corticosterone data set to include hyenas which were at least 13 months old and born after 2006. These data inclusion criteria were intended to preserve the temporal association between the early life social environment and the later life stress phenotype. We also limited our quantification of this stress phenotype to a comparable period of time when we also had behavioral and DNA methylation data (post 2006) from the 30 hyenas in the study population.

Collection and processing of key data elements

The dataset for this analysis was extracted from a rich database of demographic, behavioral and physiological information for individually identifiable hyenas. In line with our overarching hypothesis that the early social environment may affect the future stress phenotype via pathways involving changes in DNA methylation, key variables of interest included adult fecal corticosterone concentrations (the phenotype outcome of interest), the early social environment based on the hyena's mother's rank and metrics of maternal care

during the cub (early) life stage (hypothesized determinants of both DNA methylation and adult stress phenotype), genome-wide DNA methylation measured from whole blood via Enhanced Reduced Representation Bisulfite Sequencing (ERRBS), and the hyenas' age at the time of blood draw, which we included as a precision covariate in the analysis given that age is a determinant of DNA methylation (Laubach, Faulk, et al., 2019).

Adult stress phenotype: Fecal corticosterone measurement

As part of our standard protocol, we collected fecal samples from hyenas when they were observed defecating. These samples were flash frozen in the field until they were lyophilized, and steroid hormones were extracted in the U.S. Here, we focused on fecal corticosterone hormone concentrations, and the methods of this assay have been described extensively in previous publications from our lab (Dloniak et al., 2004; Van Meter et al., 2009).

Early life social environment: Maternal rank and metrics of maternal care

Maternal rank

A hyena's rank within its clan is determined based on outcomes of aggressive interactions observed during morning and evening observation periods using all-occurrence sampling (Altmann, 1974). These aggressive interactions are incorporated into a rank matrix which is used to calculate social dominance ranks for all adult females in a clan and updated annually. We normalized rank data on a scale of -1 (lowest rank) to 1(highest rank) to account for variation in clan size, and assigned rank the year in which each cub was born (Engh et al., 2000; Holekamp & Smale, 1993; Smale et al., 1993).

Maternal care

We derived metrics of maternal care from focal animal surveys (FAS) (Altmann, 1974), focusing on incidents when a mother and cub spent time in close proximity to one another (≤1m), the time a mother spent nursing her cub, and the time she spent grooming her cub. These methods have been described previously in detail (Greenberg, 2017). For this analysis, FAS data were restricted to include only sessions collected between 2012-2013, in which offspring were < 13 months of age, and their mothers were lactating. Mean weaning age in this species is 13.5 months (Holekamp et al., 1996).

Hyena age

Each hyena's age was estimated to within ± seven days based on its physical appearance and behavior when it was first observed outside of their natal den (Holekamp et al., 1996), and sex was determined based on the shape of the glans of the erect phallus once cubs were at least three months old (Frank, Glickman, & Powch, 1990).

Genome-wide DNA methylation via Enhanced Reduced Representation Bisulfite Sequencing (ERRBS)

Genomic library preparation and sequencing

We measured genome-wide DNA methylation at a single nucleotide resolution in whole blood collected from hyenas during the subadult or adult life stages (Garrett-Bakelman et al., 2015). All procedures were carried out at the University of Michigan Epigenomics Core following the protocol of Garrett-Bakelman et al. (2015).

First, we digested approximately 25-75 ng of genomic DNA for 16-18 hours with the restriction enzyme MSpI. Digested DNA was purified via phenol-chloroform extraction and ethanol precipitation. Next, we ran the digested DNA through an end-repair reaction, and an A-tailing reaction, which adenylated the 3' of the DNA. Third, we ligated paired-end methylated adapters to the A-tailed DNA and incubated at 16℃ overnight (Gu et al., 2011). Next, we selected the ligated DNA fragments in the ranges of 150-250 bp and 250-450 bp from an agarose gel and purified the excised fragments with a Qiagen QIAquick® Gel Extraction kit. Size-selected DNA was bisulfite treated using Zymo Easy DNA Methylation™ kits, followed by PCR enrichment of the bisulfite converted DNA using the Roche FastStart™ High Fidelity PCR system. Prior to sequencing, DNA libraries were cleaned using AMPure XPSPRI beads, and DNA library quantity and quality were assessed with Qubit® High Sensitivity dsDNA kit and Agilent's High Sensitivity D1000 Tape screen, respectively. Finally, we multiplexed five libraries per flow cell on an Illumina HiSeq4000® platform for single-end sequencing with a 50-nucleotide read length. Included alongside of the hyena samples were libraries for a human genomic DNA sample and a lambda phage DNA sample used as controls and to estimate bisulfite conversion efficiency. More detailed information on the ERRBS library preparation and sequencing protocol, can be found in Garrett-Bakelman et al. (2015).

Bioinformatics pipeline

We assessed the quality of raw ERRBS data and identified specific reads from each sequenced sample that required trimming using FastQC (FastQC) (v0.11.3). We used TrimGAlore (Trim Galore!) (v0.4.5) to remove low quality bases with Phred quality scores < 20,

as well as adapter sequences, primers and extra bases from the 3' ends of reads that were the product of poly-Atail end-repair. Next, we used Bismark (v0.19.0) to perform alignment and methylation calling (identification of methylated vs unmethylated CpG sites) of sequenced short reads (Krueger & Andrews, 2011). In this program, residual cytosines are converted to thymines in both the sequenced reads and the reference spotted hyena genome provided by the Bejing Genome Institute. Using Bowtie2 (v2.3.4) (Langmead & Salzberg, 2012) within Bismark, we then aligned short reads using the default parameters (multi-seed length of 20bp with 0 mismatches) and methylation calls were retained for all nucleotides with a read depth ≥ 5. Prior to downstream statistical analysis, we further restricted the data set to nucleotides with a read depth ≥ 10 and we used methylKit to exclude nucleotides that did not have coverage across all samples (Akalin et al., 2012).

Statistical analyses

Data preparation

Prior to formal analysis, we summarized and controlled for confounding variables that could bias estimates of maternal care behaviors and offspring fecal corticosterone levels (both of which are based on multiple observations or values per hyena) such that each hyena ultimately had a single value for each variable.

First, to summarize maternal care measurements, we extracted the Best Linear Unbiased Predictors (BLUPS) from a mixed-effects model. The BLUPs effectively represent each hyena's deviation in amount of maternal care received relative to the population average after accounting for key biological covariates. This procedure was previously described in detail in

Chapter 4. In brief, we used separate generalized linear mixed-effects models where counts of each maternal care behavior (number of minutes spent in close proximity, nursing, and grooming) were entered as separate dependent variables, and independent variables included the offspring's age in months, time of day (AM vs PM), status of wildebeest and zebra migration (present vs absent) on the date on which the FAS data were collected, a random intercept for individual ID, and an unstructured covariance matrix. Because there were multiple options for the type of model to use for count data, we selected the best fitting modeling using the Akaike Information Criterion (AIC) when comparing different distributions (Poisson, negative binomial 1 [variance = $\phi\mu$], and negative binomial 2 [variance = $\mu(1+\mu/k)$]) each fit with and without a zero-inflation correction. In all models, we included an offset for the total time the mother and offspring were present together during a session, such that the BLUPs represented the incident rates of behaviors for each mother offspring pair (i.e. the conditional mode of the proportion of time spent doing a behavior over the total time observed).

Similar to the maternal care metrics, we summarized repeated measures of fecal corticosterone via a mixed-effects linear regression model in which the natural log of fecal corticosterone was the dependent variables. Independent variables include age in months, reproductive state, and time of day (AM vs PM) when the fecal sample was collected, a random intercept for offspring ID to account for correlations between samples collected from the same individual, and an unstructured covariance matrix. We then calculated BLUPS from this mixed-effects model. Here again, the BLUPs effectively represent each hyena's deviation in fecal corticosterone relative to the population average after accounting for key biological covariates. This method has been previously used to consolidate repeated measurements of a given

variable into a single value per individual without making assumptions about the underlying distribution of the data (Boeke et al., 2012; Perng et al., 2012). Linear mixed-models were run using the R package lme4 (Bates, Maechler, Bolker, & Walker, 2014).

Main analysis

The main analysis sought to identify differentially methylated CpG sites in the offspring that were associated with both later life corticosterone levels and maternal social rank and/or maternal care during a hyena's first year of life (Figure 5.1.). We carried out this portion of the analysis in two steps, as described below.

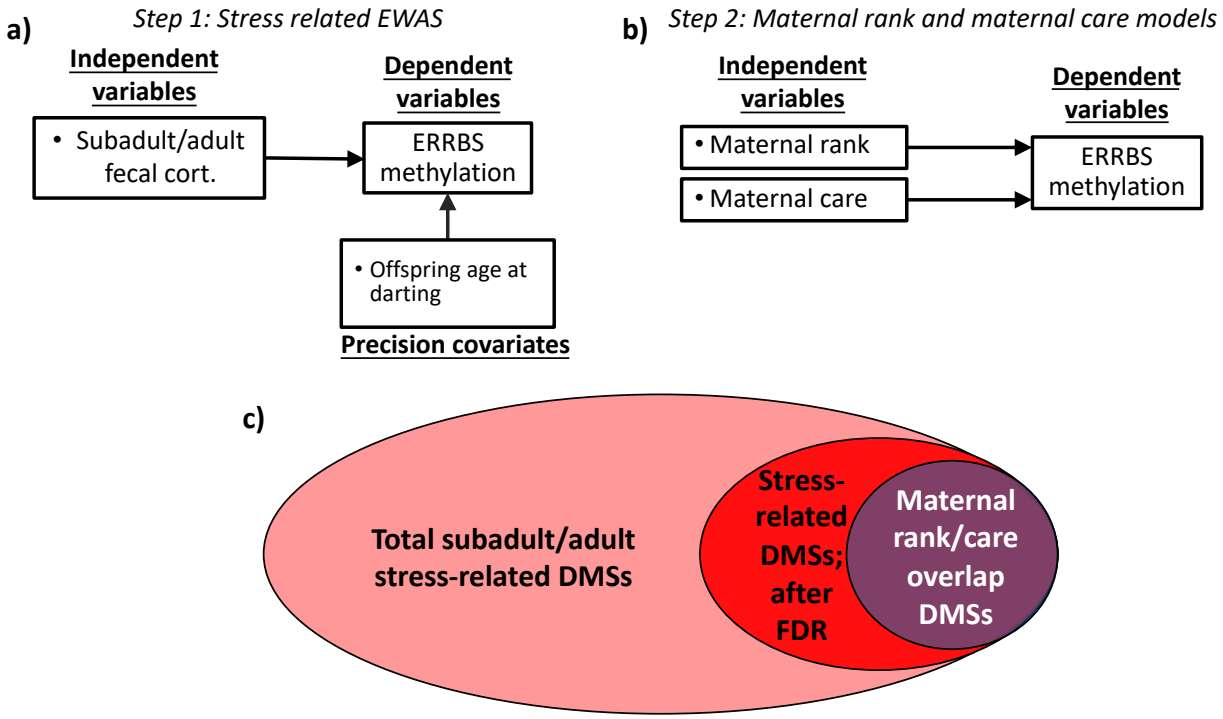

Figure 5.1. Conceptual models a) of the epigenome-wide association study (EWAS) for identification of differentially methylated sites (DMSs) from enhanced reduced representation bisulfite sequencing (ERRBS) data that are associated with subadult/adult stress phenotype (quantified as fecal corticosterone BLUPs), b) conceptual models of maternal rank and maternal care associations with DMSs identified in step 1, c) and a Venn diagram of potentially mediating DMSs (in purple).

Step 1: Identify CpG sites associated with later life stress phenotype via an epigenome-wide association study (EWAS)

We conducted the EWAS using the program MACAU (Lea, Tung, & Zhou, 2015), which fits binomial regression models for each sequenced CpG site using the counts of total and methylated CpG sites. We modeled offspring fecal corticosterone BLUPs as the independent variable of interest and controlled for offspring age in months as a covariate. We then corrected for multiple comparisons using Benjamini-Hochberg false discovery rate (FDR)(Hochberg & Benjamini, 1990) of 5%, and identified CpG sites that were differentially-methylated with respect to fecal corticosterone levels during the later life stage. For all DMSs that passed the FDR threshold, we copied the hyena DNA sequence containing the CpG site of interest, approximately 150bp upstream and downstream of the target, and saved the short sequences as Fasta files. Given that the draft spotted hyena genome is not annotated, we mapped each DNA sequence containing the CpG site of interest back to the human genome (UCSC Human Dec. 2010 [GRCh38/hg38] Assembly) using the UCSC Genome Browser tool, the BLAST-like alignment tool (BLAT) (W. J Kent et al., 2002).

Step 2: Identify CpG sites associated with maternal rank and maternal care metrics

Among the differentially methylated sites (**DMS**s) identified in Step 1, we sought to further identify those that are also associated with maternal rank and maternal care metrics. By identifying CpG sites that were differentially methylated with respect to later life fecal

corticosterone *and* associated with early life maternal rank/care metrics, we effectively identified potential mediating biomarkers of stress-related genomic pathways.

To accomplish this, we ran separate generalized linear regression models for each previously identified stress related DMS, and we modeled the outcome as the methylated read counts of at each site. In these models the independent variable was maternal rank or individual maternal care metrics. Also, we treated the total read counts at each DMS as an offset and assumed an underlying Poisson distribution. We considered a CpG site to be relevant based on two criteria. First, the beta coefficient for the relationship between a given early life social characteristic and DNA methylation for the CpG site of interest needed to reach statistical significance at a nominal *P*-value cut-off of <0.1. Second, given our overarching hypothesis that better quality or quantity of maternal care is protective against an adverse stress phenotype in adulthood, we also considered the direction of effects from both the stress related EWAS and the maternal rank / maternal care generalized linear regression models. Specifically, we expected that a positive association between fecal corticosterone and CpG methylation would correspond to an inverse association between early life social metrics and CpG methylation and vice versa. This expectation was based on our previous work (Chapter 4), which showed that higher quantity of maternal care was associated with lower baseline fecal corticosterone in adults.

RESULTS

We analyzed data from 29 female hyenas which had an average ± SD age of 18.8 ± 3.9 months on the date their DNA methylation was assessed. After processing the data through the

bioinformatic pipeline, we quantified DNA methylation at 2,246,867 unique CpG sites that had a minimum of 10x coverage in all 29 hyenas. The average bisulfite conversion rate was 99.4% (SD ± 0.1%) and the average alignment efficiency of reads to the draft spotted hyena genome was 75.5% (SD ± 2.6) for the 29 hyena samples. Of the 29 hyenas for which we had ERRBS data, we also had fecal samples from 25 of them (median of 3 samples per hyena). Mean ± SD later life corticosterone concentration in our study population was 72.9 ± 62.8 ng/g. During the FAS data collection, we observed mother-offspring pairs together on average for 26 minutes per FAS session, with a median of 2.5 sessions recorded per pair. In this subsample, mothers spent an average of 79.8% (SD ± 30.0%) of their time in close proximity to offspring, 66.4% (SD ± 31.2%) of time nursing, and 4.9% (± 7.3%) of time grooming.

The uncorrected EWAS revealed 97,745 stress related differentially methylated CpG sites. The distribution of the observed p-values generated from the binomial regression based EWAS did not indicate any substantial outliers and generally conformed with the expected p-value distribution (Figure 5.2.a). Additionally, the p-values from the EWAS generally followed a uniform distribution (Figure 5.2.b). After applying Benjamini-Hochberg's FDR correction of 5%, 15 DMSs were significantly associated with fecal corticosterone (Figure 5.3., Table 5.1.).

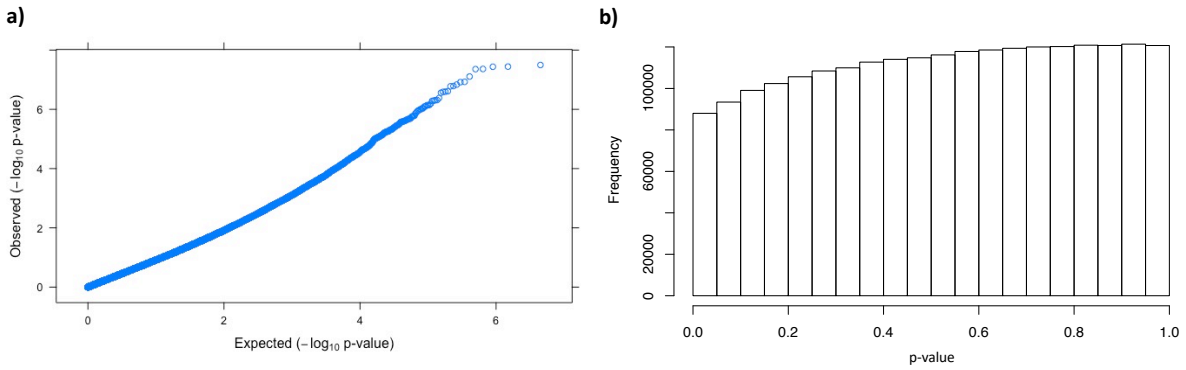

Figure 5.2. Diagnostic plots for stress related EWAS. a) A QQ-plot showing the observed $-\log_{10}$ p-values versus the expected $-\log_{10}$ p-values. b) A frequency histogram showing a relatively uniform distribution of p-values generated from the EWAS.

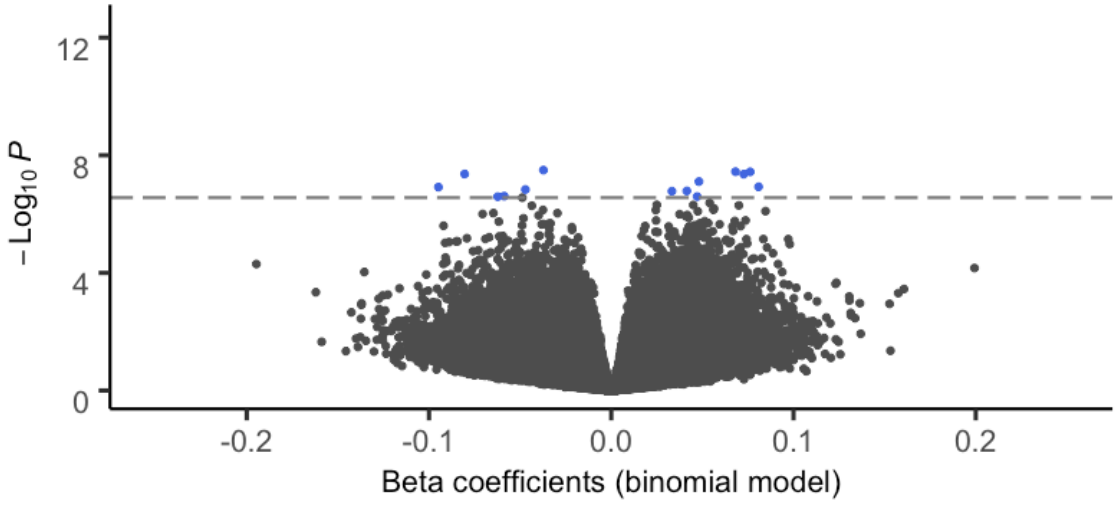

Figure 5.3. Volcano plot of the $-\log_{10}$ p-values by the beta coefficients from a binomial regression epigenome wide association study (EWAS). Counts of methylated over total cytosines from enhanced reduced representation bisulfite sequencing data were modeled as a function of variation in subadult and adult spotted hyena fecal corticosterone. The horizontal line represents the Benjamini-Hochberg false discovery rate (FDR) cutoff of 0.05%, above which the blue points are significant.

DMSs (CpG site location *C. crocuta* genome)	β ± SE [†]	FDR p-value [‡]	Unadjusted p-value	Acceptance rate	Closest human gene(s) (BLAT) [§]	Gene position (hg38)	Chromosome (hg38)	BLAT score/% identical match/# bp span [§]	Gene full name (hg38)
C26291268 87	-0.049 ± 0.009	0.041	2.76E-07	0.471	*IQSEC1*	gene body	chr3	86 / 91.4% / 104	IQ motif and Sec7 domain 1
scaffold110 2814293	0.081 ± 0.015	0.034	1.18E-07	0.480	*SFI1*	gene body	chr22	25 / 81.5% / 30	SFI1 centrin binding protein
scaffold13 254293	**-0.062 ± 0.012**	**0.041**	**2.57E-07**	**0.449**	***VPS13D***	**gene body**	**chr1**	**58 / 94.0% / 66**	**vacuolar protein sorting 13 homolog D**
scaffold139 1925966	-0.095 ± 0.018	0.034	1.21E-07	0.488	*EPN2*	gene body	chr17	173 / 93.5% / 210	epsin 2
scaffold147 1445632	**-0.047 ± 0.009**	**0.034**	**1.46E-07**	**0.495**	***LIN28B***	**gene body**	**chr6**	**193 / 89.8% / 261**	**lin-28 homolog B**
scaffold193 6562606	0.073 ± 0.013	0.020	4.40E-08	0.464	*MFSD2A*	gene body	chr1	32 / 100.0% / 99	major facilitator superfamily domain containing 2A
scaffold207 3315565	0.076 ± 0.014	0.020	3.66E-08	0.476	*GTF2IRD1*	gene body	chr7	26 / 100.0% / 39	GTF2I repeat domain containing 1
scaffold248 5799761	**0.047 ± 0.009**	**0.041**	**2.54E-07**	**0.456**	***NDRG3***	**<100 bp from exon 1**	**chr20**	**37 / 95.3% / 92**	**NDRG family member 3**
scaffold322 4736152	0.041 ± 0.008	0.034	1.64E-07	0.531	*TMPRSS9*	gene body	chr19	24 / 88.0% / 25	transmembrane serine protease 9
scaffold334 155724	-0.080 ± 0.015	0.020	4.34E-08	0.434	*IL1RAPL2*	gene body	chrX	80 / 72.5% / 187	interleukin 1 receptor accessory protein like 2
scaffold364 1284416	**0.048 ± 0.009**	**0.029**	**7.82E-08**	**0.460**	***WNTB2B***	**gene body**	**chr1**	**42 / 85.2% / 55**	**Wnt family member 2B**
scaffold443 1451106	-0.059 ± 0.011	0.041	2.45E-07	0.465	*AC005258.1*	gene body	chr19	242 / 90.9% / 296	cDNA, FLJ99163 (of the GXGD family of apsartic proteases)
scaffold466 860429	**0.033 ± 0.006**	**0.034**	**1.67E-07**	**0.478**	***TTC13***	**gene body**	**chr1**	**31 / 86.2% / 36**	**tetratricopeptide repeat domain 13**
scaffold506 286735	0.068 ± 0.012	0.020	3.61E-08	0.437	*PACS2*	gene body	chr14	22/ 100.0% / 26	phosphofurin acidic cluster sorting protein 2
scaffold61 9436494	-0.037 ± 0.007	0.020	3.19E-08	0.470	*MBD3*	gene body	chr19	22 / 95.9% / 29	methyl-CpG binding domain protein 3

[†] Untransformed (natural log scale) beta estimates are from binomial regression models run in program MACAU.

[‡] FDR p-value based on Benjamini-Hochberg correction (0.05%)

[§] Mapping of spotted hyena DNA sequences onto the human genome (Human Dec. 2013 [GRCh38/hg38] Assembly) was done using the top hit from the UCSC Genome Browser's BLAT tool.
All bold DMSs were significantly associated with a matneral care metric at $P<0.1$.

Table 5.1. Epigenome wide association results showing differentially methylated CpG sites (DMSs) by fecal corticosterone BLUPs among 25 female spotted hyenas. DNA methylation was assessed during the subadult life stage, approximately, and fecal corticosterone samples were collected from animals during the subadult and adult life stages.

Closest human gene(s) (BLAT) [§]	DMSs (CpG site location *C. crocuta* genome)	Maternal rank [†]		Close proximity [‡]		Nursing [‡]		Grooming [‡]		Direction of βs matches prediction [¶]
		β ± SE	Unadjusted p-value	β ± SE	Unadjusted p-value	β ± SE	Unadjusted p-value	β ± SE	Unadjusted p-value	
IQSEC1	C26291268 87	-0.18 ± 0.49	0.708	1.07 ± 11.00	0.923	15.15 ± 10.77	0.159	0.20 ± 7.53	0.979	
SFI1	scaffold110 2814293	0.00 ± 0.06	0.983	0.07 ± 0.70	0.918	-0.11 ± 0.70	0.873	-0.03 ± 0.41	0.944	
VPS13D	**scaffold13 254293**	0.44 ± 0.37	0.233	11.49 ± 7.63	0.132	**9.10 ± 5.20**	**0.080**	**6.24 ± 2.33**	**0.007**	yes
EPN2	scaffold139 1925966	-0.52 ± 0.70	0.456	-12.84 ± 13.27	0.335	16.53 ± 15.18	0.276	3.02 ±. 7.44	0.685	
LIN28B	**scaffold147 1445632**	-0.14 ± 0.28	0.620	1.10 ± 3.96	0.781	3.98 ± 4.80	0.408	**3.82 ± 2.06**	**0.063**	yes
MFSD2A	scaffold193 6562606	0.00 ± 0.06	0.960	0.16 ± 0.71	0.819	-0.24 ± 0.84	0.777	-0.03 ± 0.49	0.948	
GTF2IRD1	scaffold207 3315565	0.00 ± 0.06	0.973	0.19 ± 0.75	0.803	-0.17 ± 0.90	0.854	-0.06 ± 0.51	0.913	
NDRG3	**scaffold248 5799761**	0.71 ± 0.48	0.145	**18.04 ± 7.49**	**0.016**	4.91 ± 4.21	0.243	-4.86 ± 4.39	0.267	no
TMPRSS9	scaffold322 4736152	0.01 ± 0.05	0.751	0.19 ± 0.61	0.760	-0.46 ± 0.75	0.539	-0.19 ± 0.48	0.689	
IL1RAPL2	scaffold334 155724	-0.04 ± 0.05	0.479	-0.31 ± 0.70	0.663	-0.81 ± 0.82	0.319	0.00 ± 0.49	0.998	
WNTB2B	**scaffold364 1284416**	0.88 ± 0.57	0.119	**20.08 ± 10.62**	**0.059**	4.35 ± 6.08	0.475	-50.81 ± 56.23	0.366	no
AC005258.1	scaffold443 1451106	0.00 ± 0.04	0.988	-0.11 ± 0.63	0.863	-0.01 ± 0.68	0.988	0.07 ± 0.41	0.858	
TTC13	**scaffold466 860429**	-0.03 ± 0.18	0.869	2.11 ± 2.50	0.398	-0.17 ± 2.88	0.954	**-6.29 ± 3.47**	**0.070**	yes
PACS2	scaffold506 286735	0.00 ± 0.05	0.965	0.14 ± 0.65	0.836	-0.07 ± 0.66	0.914	-0.02 ± 0.38	0.968	
MBD3	scaffold61 9436494	0.01 ± 0.03	0.813	-0.16 ± 0.42	0.704	-0.02 ± 0.52	0.969	0.01 ± 0.35	0.973	

[†] Untransformed (natural log scale) beta estimates are from zero inflated poisson regression model in which methylated read counts are the outcome and total read counts is included as an offset; n = 27.

[‡] Untransformed (natural log scale) beta estimates are from zero inflated poisson regression model in which methylated read counts are the outcome and total read counts is included as an offset; n = 12.

[§] Mapping of spotted hyena DNA sequences onto the human genome (Human Dec. 2013 [GRCh38/hg38] Assembly) was done using the top hit from the UCSC Genome Browser's BLAT tool.

[¶] The beta estimates from the EWAS, in which fecal corticoserone was the predictor, and from Poisson regression models, in which maternal rank and maternal care variables were the predictors, were expected to be in opposite directions; 'yes' demarks where this expectation was met.

All bold DMSs were significantly associated with a matneral care metric at $P<0.1$.

Table 5.2. Associations of differentially methylated CpG sites (DMSs) by maternal rank and maternal care variables among the 15 DMSs identified in the stress phenotype EWAS for 12 female spotted hyenas that had no missing data (maternal rank/care, ERRBS, and fecal corticosterone variables).

When we examined associations between maternal rank and maternal care metrics and DNA methylation of the 15 CpG sites identified in the EWAS, five CpG sites were significantly differentially methylated with respect to the maternal care variables. Of these five, three exhibited the inverse direction of associations that we expected (i.e., given that maternal care is hypothesized to be protective against an adverse stress phenotype as indicated by fecal corticosterone levels, then CpG sites that are positively associated with fecal corticosterone should be inversely related to maternal care and vice versa). The proportion of time that cubs were groomed and nursed by their mothers were each positively associated with DNA methylation at CpG site scaffold13 254293 (P = 0.007 and P = 0.080 for grooming and nursing, respectively) Table 5.2.), and fecal corticosterone was negatively associated with DNA methylation at this DMS (P = 0.041; Table 5.1.). This CpG site is located near the human vacuolar protein sorting 13 homolog D (*VPS13D*) gene (W. J Kent et al., 2002; W. James Kent, 2002). We also observed a positive association of time spent grooming that approached significance (P = 0.063; Table 5.2.) and a negative association of fecal corticosterone (P = 0.034; Table 5.1.) with respect to DNA methylation at a CpG site scaffold147 1445632 (P = 0.063; Table 5.2.). This DMS aligned near the lin-28 homolog B (*LIN28B*) gene in humans (W. J Kent et al., 2002; W. James Kent, 2002). Finally, grooming was negatively associated with DNA methylation of the CpG site scaffold466 860429 (P = 0.070; Table 5.2.), and fecal corticosterone was positively associated with DNA methylation (P = 0.034; Table 5.1.) at this DMS, which aligns near the tetratricopeptide repeat domain 13 (*TTC13*) gene in humans (W. J Kent et al., 2002; W. James Kent, 2002).

Of note, there were two DMSs that were associated with maternal care metrics and fecal corticosterone, but the directions of the associations were the same, thereby precluding potential mediation based on our *a priori* hypothesis. The time mother-offspring pairs spent in close proximity was positively associated with offspring DNA methylation ($P = 0.016$; Table 5.2.), as well as with fecal corticosterone ($P = 0.041$; Table 5.1.) at CpG site scaffold248 5799761. We also observed a positive association of time spent in close proximity that approached significance ($P = 0.059$; Table 5.2.) and a positive association fecal corticosterone ($P = 0.029$; Table 5.1.) with DNA methylation at the CpG site scaffold364 1284416. Maternal rank during the year in which the offspring was born was not related to any of the stress related DMSs identified in this sample of female hyenas (Table 5.2.).

DISCUSSION

In this exploratory analysis of 29 female spotted hyenas, we employed a two-step approach to identify differentially methylated genomic regions that might link hyenas' early life social environment to their future stress phenotypes. The first step of the analysis, which was an EWAS with adult fecal corticosterone as the outcome of interest, revealed multiple differentially methylated regions occurring near genes that have been functionally implicated in the stress response. In the second step of the analysis, we examined associations between maternal rank or maternal care metrics with DNA methylation of CpG sites identified in Step 1 in order to home in on specific regions of the genome related to both the early social environment and adult stress phenotype. We found three differentially methylated regions of interest: one located near the human *VPS13D* gene; one near *LIN28B*; and one near the *TTC13*.

Contrary to our expectations, none of the DMSs that were associated with adult fecal corticosterone levels were associated with maternal rank during the year in which a hyena was born. We discuss results in greater detail below.

Comparability of EWAS metrics to that of other mammals

Our EWAS results are comparable to those of studies in other mammals that involved use of an ERRBS protocol. For example, bisulfite conversion rates of spotted hyena, human, and yellow baboon were all ≥ 99.4% (Garrett-Bakelman et al., 2015; Lea, Altmann, Alberts, & Tung, 2016). The numbers of uniquely aligned sequencing reads in hyenas were approximately double the numbers reported for humans, baboons, and canids (domestic dogs and wolves), but hyena versus human read mapping alignment efficiencies were comparable at around 70% (Garrett-Bakelman et al., 2015; Janowitz et al., 2016; Lea et al., 2016). Considering differences between the ERRBS (Garrett-Bakelman et al., 2015) protocol and the RRBS (Meissner et al., 2005) protocol, numbers of samples pooled together in sequencing libraries between studies, and the use of different versions of the Illumina sequencing platform, the ERRBS data from hyenas seem comparable to those from other mammals.

Biological interpretation of CpG sites of interest

We identified 15 DMSs that were significantly associated with adult fecal corticosterone concentrations after FDR correction, five of which were also associated with early life maternal care metrics. Three of these DMS showed the predicted inverse direction of association when comparing the beta estimates from maternal care metric models with fecal corticosterone

models. Focusing on these three candidate DMSs which meet the criteria for mediating biomarkers of stress related phenotypes, we discuss the direction of the associations of each DMS with respect to fecal corticosterone levels and early life maternal care metrics as well as their potential biological function, below.

More time spent being groomed and nursing as a cub, and lower adult fecal corticosterone levels corresponded with greater DNA methylation of a CpG site in the hyenas that mapped near the human *VPS13D* gene. The VPS13 family of proteins are widely conserved, and function in a number of basic cellular processes (Bateman, 2019; Brown et al., 2015). In humans, *Drosophila*, and yeast, *VPS13* gene homologs contribute to the structural organization and the function of mitochondria in addition to playing a role in transporting molecules from the golgi apparatus to endosomes and lysosomes (Anding et al., 2018; Muñoz-Braceras, Tornero-Écija, Vincent, & Escalante, 2019; Park et al., 2016; Yeshaw et al., 2019). Of more relevance to stress phenotypes, *in vitro* treatment of lymphoblastoid cells with both cytokines and corticosteroids revealed differences in interleukin-6 (IL-6) production, corresponding to a SNP in the in *VPS13D* gene (Nakada et al., 2015). Although we have only correlative evidence, the identification of a potential molecular biomarker in hyenas near the human homolog *VPS13D* suggests that inflammation pathways involving IL-6 may be important for understanding the relationship between early life maternal care and later life stress phenotypes.

We also found that greater proportion of time grooming early in life and lower fecal corticosterone later in life corresponded with greater DNA methylation of a CpG site that mapped near the human *LIN28B* gene among hyena offspring. In a rat model of depression,

animals bred for depressive phenotypes exhibited higher expression of *LIN28B* and *IL6* mRNA, which suggests that LIN28B expression acts on upstream elements in the *IL6* regulatory pathway (Wei et al., 2016). Elevated inflammation biomarkers, like IL-6, are commonly found in animals and humans that are chronically stressed and depressed, respectively (Dowlati et al., 2010; Sukoff Rizzo et al., 2012). Additionally, two recent meta-analyses of genome-wide association studies identified *LIN28B* as having a role in risk of depression in multiple large human cohorts (Howard et al., 2019; Hyde et al., 2016). Finally, genetic variation of the *LIN28B* gene is associated with age at menarche (Ong et al., 2009; Perry et al., 2014). Early life social stressors have also been found to be associated with age of menarche (Boynton-Jarrett et al., 2013; Henrichs et al., 2014), which in turn was associated with depressive symptoms (Galvao et al., 2014; Sequeira, Lewis, Bonilla, Smith, & Joinson, 2017), suggesting that a pathway involving the *LIN28B* gene may link early life social environment, growth and development, and stress phenotypes.

Lastly, we found that less grooming and higher fecal corticosterone were related to greater DNA methylation of a CpG site near the human *TTC13* gene. However, little is known about the function of the *TTC13* gene other than that it is a protein coding gene (Bateman, 2019; Brown et al., 2015).

Strengths and limitations

Strengths of this study are the novel and gregarious study system for which we have rich data on multiple metrics of the early social environment, and our ability to examine the entire early social environment → DNA methylation → adult stress phenotype pathway in the same

study population – an endeavor that has generally been completed in piecemeal fashion in other study systems.

However, our study is not without limitations. First, our relatively small sample size of 29 individuals may have limited our ability to detect significant associations. This was particularly true for the second step of the analysis in which we looked at associations between maternal care variables and differential methylation of CpG sites identified from the EWAS. Second, while we had a draft hyena genome, it was not annotated. Therefore, we relied on the distantly related but well annotated human genome to align the DMSs and make inferences about the locations of those DMSs based on nearby human genes, which is prone to some amount of inaccuracy. Third, many of the CpG sites for which we quantified DNA methylation, including those identified in the EWAS, had average DNA methylation values across individuals near 0% or 100%. Thus, even though we used a strict FDR cutoff, we cannot rule out the possibility of false positive hits. Finally, fecal corticosterone is a highly variable measure of the stress phenotype. We suggest that future studies consider additional stress related measures including behavioral measures of stress. In addition, investigating life-history and fitness related traits would provide deeper insights into ultimate explanations regarding the developmental effects of early life social experience and the functional molecular pathways upon which natural selection acts.

CONCLUSIONS

Our results suggest that specific aspects of maternal care (i.e. amount of time spent grooming and nursing) are associated with differential methylation of three CpG sites identified

in our stress related EWAS. The DMSs identified in both steps of our analysis are of particular interest for understanding the etiology of adult stress phenotypes that develop in response to variable early life social environments and provide novel insights into a plausible molecular mechanism.

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