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Early life social and ecological determinants of global DNA methylation in wild spotted hyenas

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Abstract

Environmental factors early in life can have lasting influence on the development and phenotypes of animals, but the underlying molecular modifications remain poorly understood. We examined cross-sectional associations among early life socioecological factors and global DNA methylation in 293 wild spotted hyenas (*Crocuta crocuta*) in the Masai Mara National Reserve, Kenya, grouped according to three age classes (cub, subadult and adult). Explanatory variables of interest included annual maternal rank based on outcomes of dyadic agonistic interactions, litter size, wild ungulate prey density and anthropogenic disturbance in the year each hyena was born based on counts of illegal livestock in the Reserve. The dependent variable of interest was global DNA methylation, assessed via the LUminometric Methylation Assay, which provides a percentage 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 3 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.

KEYWORDS
Developmental Origins of Health and Disease, DNA methylation, mammals, social environment

1 | 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, Hofman, & Hanson, 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, Faulk, Cardenas, & Perng, 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).

1.1 | 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; 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 (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 levels 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, Tung, Archie, & Alberts, 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 lifespan (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).

1.2 | 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 greater 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 a larger magnitude of associations during earlier than later age classes given that explanatory variables were measured during a hyena's birth year.

2 | METHODS

2.1 | 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, 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, Smale, & Szykman, 1996; Holekamp et al., 2012; Smith, Memenis, & Holekamp, 2007). Female hyenas typically give birth to one or two offspring (Frank, Glickman, & Licht, 1991; Holekamp et al., 1996), which depend on their mothers for food and protection until offspring are approximately 2 years of age (Watts, Tanner, Lundrigan, & Holekamp, 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, behavioural 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 behavioural 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 Appendix S1). Of these individuals, 58 had repeated measures capturing more than one age class due to the opportunistic nature of immobilizations and blood draws.

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

2.2.1 | 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 a singleton or twin litter when it was first seen above ground.

2.2.2 | 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 analysed by Green, Johnson-Ulrich, Couraud, and Holekamp (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 numbers 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 (our personal observations).

Prey density during discrete developmental periods

Twice each month, research assistants counted all prey animals observed within 100 m 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 periconceptional period (1.5 months before and 1.5 months after conception), during gestation (3 months prior to birth), and 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 modelling 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, Roseboom, & Bleker, 2005).

2.2.3 | 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 birth dates based on their physical appearance when first observed as infants. Based on their pelage, morphology and behaviour, 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 three-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).

2.3 | Dependent variable: global DNA methylation

2.3.1 | 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 the 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 there was potential variation in DNA quality due to storage time.

2.3.2 | Global DNA methylation assay

We quantified global DNA methylation as percentage methylated CCGG sites (%CCGG methylation) in peripheral leukocytes using a LUMA assay (Karimi, Johansson, & Ekström, 2006; Karimi, Johansson, Stach, et al., 2006). Extensive details on our laboratory methods and QA/QC are included in the Appendix S1. 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 (<1 kb) transcription start sites, 45% are in gene bodies and 52% are in noncoding repetitive elements (Ball et al., 2009; Kinney et al., 2011). Given the high proportion of CpG sites within gene bodies and noncoding 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 favourable outcome than lower %CCGG methylation.

2.4 | 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 the 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 (ICCs) 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, Forster, & Roth, 2016) and age (Bjornsson et al.., 2008) can potentially alter the relationship between early exposures and DNA methylation, we assessed for an 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 <.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

TABLE 1 Life history and social characteristics of 293 spotted hyenas as well as ecological measures from the Masai Mara, Kenya

	N[a]	%
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[b]		
Low	102	36
Medium	96	34
High	87	30
Average prey density during discrete developmental periods (per 1 km^2)[c]	***N***[a]	**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

[a]In total, 320 measurements from 293 individual hyenas; numbers may not add up to 293 individuals, due to missing values.
[b]Human presence was determined by counts of livestock within the reserve boundary and proximity to Masai villages.
[c]Prey species include the five most commonly consumed wild ungulates: impala, Thomson's gazelle, topi, plains zebra and wildebeest.

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 < .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.

2.4.1 | 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 based on the time order of years during the project.

3 | RESULTS

3.1 | Descriptive statistics

Slightly more than half the study population were females (56%), and we had more samples from individuals at older than young life

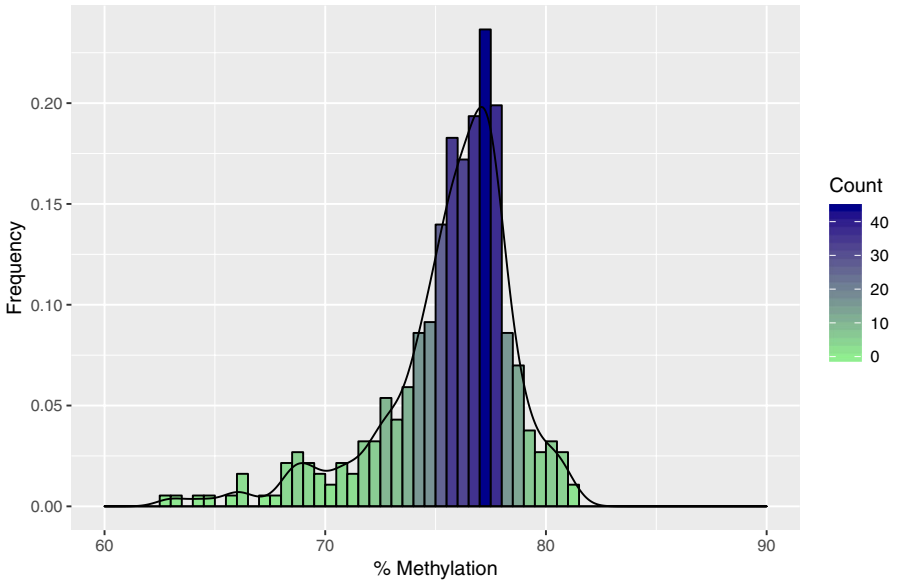

FIGURE 1 Frequency distribution of %CCGG methylation, our continuous outcome of interest, for hyena genomic DNA samples that were extracted from whole blood from 293 individual hyenas and assayed with LUMA [Colour figure can be viewed at wileyonlinelibrary.com]

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 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 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% confidence interval, CI: −0.93, 0.33], p = .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 = .02).

3.2 | 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 is 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 = .06) but not sex (p-interaction = .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 2), we binned standardized maternal rank into quartiles, with the first quartile representing the lowest maternal rank and the fourth quartile representing the highest maternal rank. 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 (Table S1 in Appendix S1).

3.3 | Cub models

Table 2 shows the Model 1 adjusted associations between explanatory variables and %CCGG methylation in hyenas during the cub life stage (for unadjusted estimates, see Table S2 in Appendix S1). 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 = .016), 3.46 (95% CI: 0.96, 5.97; p = .009) and 1.68 (95% CI: −0.96, 4.32; p = .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 = .004) %CCGG higher methylation and cubs born into high disturbance groups had 3.51 (95% CI: 0.83, 6.19; p = .013) %CCGG higher methylation. On the other hand, the 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 = .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 = .004) lower %CCGG methylation. Adjusting for each 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 = .009), and from birth to 3 months (−1.40 [95% CI: −2.44, −0.36]; p = .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).

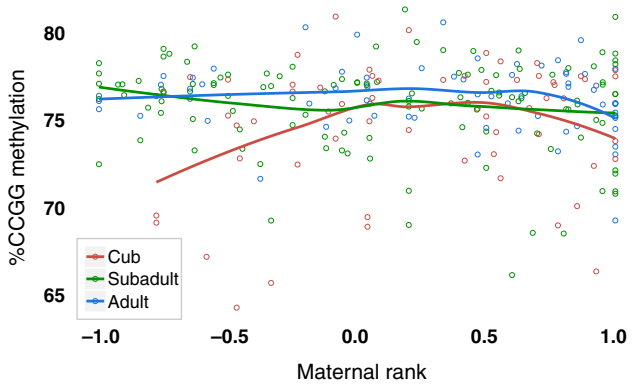

FIGURE 2 Hyena %CCGG methylation by standardized maternal rank (−1 lowest rank, and 1 highest rank) and stratified by age categories of cubs, subadults and adults [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | Subadult models

Table 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 = .201) and 2.05 (95% CI: 0.85, 3.25; p = .001) higher %CCGG methylation, respectively. We also noted a trend toward (1.26; 95% CI: −0.17, 2.69; p = .088) lower %CCGG methylation in twin than singleton litters.

TABLE 2 Model 1 adjusted associations of explanatory variables with global DNA methylation in hyenas assessed at each age category

	β (95% CI) for %CCGG methylation					
	Cub models[a]	*p*	Subadult models[a]	*p*	Adult models[a]	*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)**	**.016**	−0.50 (−1.78, 0.78)	.444	0.52 (−0.85, 1.88)	.461
Q3	**3.46 (0.96, 5.97)**	**.009**	−0.47 (−1.78, 0.85)	.486	0.81 (−0.59, 2.21)	.261
Q4 (Highest)	1.68 (−0.96, 4.32)	.217	−0.72 (−2.06, 0.62)	.296	−0.64 (−1.94, 0.65)	.333
Litter size						
Singleton	0.00 (Reference)		0.00 (Reference)		0.00 (Reference)	
Twins	0.78 (−1.42, 2.98)	.490	*−1.26 (−2.69, 0.17)*	*.088*	−0.08 (−1.51, 1.35)	.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)**	**.004**	0.84 (−0.44, 2.13)	.201	0.54 (−0.56, 1.63)	.340
High	**3.51 (0.83, 6.19)**	**.013**	**2.05 (0.85, 3.25)**	**.001**	0.80 (−0.49, 2.09)	.229
Prey density during discrete developmental periods (per 1 *SD*)[b,c]						
Periconception	**−1.18 (−2.03, −0.33)**	**.009**	0.03 (−0.53, 0.60)	.906	−0.02 (−0.51, 0.46)	.928
Gestation	−0.41 (−1.17, 0.34)	.287	0.30 (−0.59, 1.18)	.513	−0.05 (−0.55, 0.46)	.861
Birth to <3 months of age	**−1.40 (−2.44, −0.36)**	**.011**	−0.37 (−0.93, 0.19)	.197	**−0.68 (−1.14, −0.22)**	**.005**
3 to <6 months of age	0.55 (−0.51, 1.61)	.315	0.32 (−0.19, 0.83)	.220	0.15 (−0.42, 0.72)	.608
6 to <9 months of age	0.01 (−0.96, 0.97)	.991	0.01 (−0.70, 0.72)	.978	−0.06 (−0.55, 0.43)	.806

Note: Bold estimates are significant at $p < .05$, and italicized estimates are significant at $p < .1$.
[a]Models are adjusted for hyena age at blood collection (months) and sex.
[b]Prey species include the five most commonly consumed wild ungulates: impala, Thomson's gazelle, topi, plains zebra and wildebeest.
[c]Models are adjusted for prey densities from previous developmental periods.

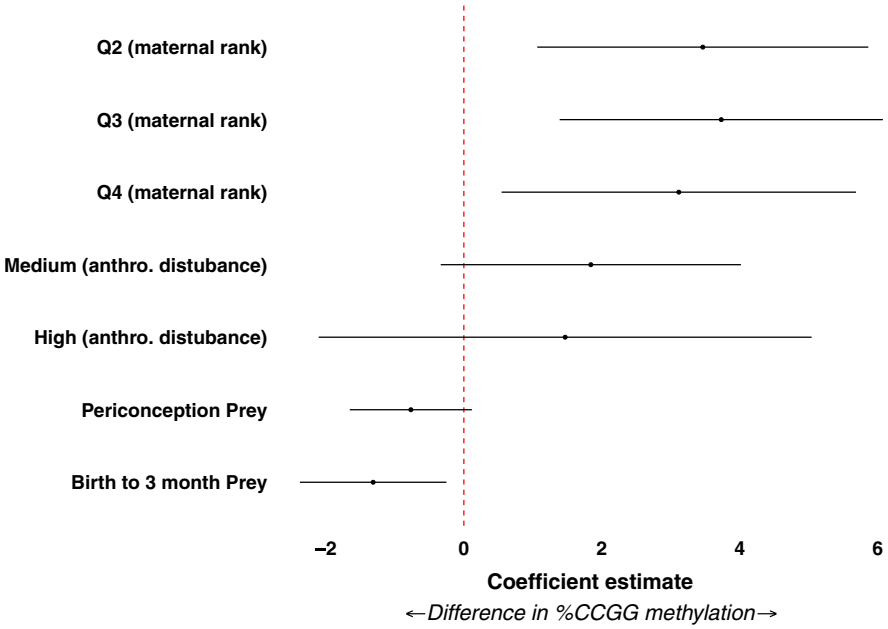

FIGURE 3 Beta estimates and 95% confidence intervals for %CCGG methylation from Model 2 in cubs. Model 2 included all significant explanatory variables from Model 1 including: maternal rank, anthropogenic disturbance, wild ungulate prey density at periconception and from birth to 3 months, as well offspring age at the time of darting and sex [Colour figure can be viewed at wileyonlinelibrary.com]

3.5 | Adult models

In the last column of Table 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 (Table S3 in Appendix S1). 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 to 0.68 (95% CI: 0.22, 1.14; p = .005) lower %CCGG methylation. None of the other early life social or ecological variables were related to global DNA methylation in this age group.

3.6 | Sensitivity analyses

Results from models where we further adjusted for sample age as a covariate were similar to those without sample age adjustment (Table S4 in Appendix S1). 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 (Li, Pan, et al., 2018), serve as the impetus for us to focus the discussion of results on models that do not include sample age.

4 | 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 percentage 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 periconceptional period through the first 3 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.

4.1 | 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).

4.2 | 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-ranking 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., 2018). 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., 2012) 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. (2013) 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 USA. However, the authors did find that attained wealth, a socioeconomic asset accrued across the lifespan, 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 probably vary not only across different life stages, but also with respect to detectable differences in DNA methylation assessed at specific loci versus at the global level. Furthermore, 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).

4.3 | 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 the 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.

4.4 | Prey density

We observed an inverse association between prey density in the first 3 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 (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 (e.g., Holekamp et al., 2012). Furthermore, we found that faecal 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 as assessed via a LUMA assay (Crudo et al., 2012). If a more powerful analysis reveals that pregnant female hyenas have elevated glucocorticoids during periods of higher than lower prey abundance, 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., 2008; Tobi et al., 2009).

4.5 | 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.

4.6 | 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 metadata on the demographic, behavioural 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 of 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 such as 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 (a) 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); (b) 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 (c) 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, such as 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 the risk of type 2 error at the cost of reducing type 1 error (Rothman, 1990).

4.7 | Conclusions

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 to higher global DNA methylation in cubs and subadults. Finally, the 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, Zhang, 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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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 the 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.

DATA AVAILABILITY STATEMENT

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.

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