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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 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 % 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.

29

30   **INTRODUCTION**

31   The Developmental Origins of Health and Disease (DOHaD) hypothesis suggests that

32   environmental conditions over the course of ontogeny have lasting effects on an organism's

33   phenotype (Gillman, 2005). Of particular interest in DOHaD are vulnerable developmental

34   stages ("sensitive periods") marked by high phenotypic plasticity (Heindel & Vandenberg, 2015)

35   such as the periconceptional period, gestation, and the early post-natal period (Gluckman,

36   Cutfield, Hofman, & Hanson, 2005; Hanson & Gluckman, 2014). The central premise of DOHaD

37   is that environmental factors, two of the most widely studied being social experiences (Loi, Del

38   Savio, & Stupka, 2013) and nutrition (Laubach, Faulk, Cardenas, & Perng, 2017), during sensitive

39   periods of development have a larger impact on phenotypes than those occurring during other

40   life stages (Bateson et al., 2004; Ben-Shlomo & Kuh, 2003).

41   **DNA methylation as a pathway of DOHaD phenomena**

42   One molecular pathway hypothesized to underlie DOHaD phenomena involves DNA

43   methylation (Waterland & Michels, 2007). Among mammals, DNA methylation primarily refers

44   to a methyl group that is covalently bonded to the fifth carbon of a cytosine base found

45   primarily in cytosine-phosphate-guanine (CpG) dinucleotide pairs (Razin & Riggs, 1980). Of

46   particular relevance to DOHaD is the fact that DNA methylation is a well-characterized and

47   mitotically stable epigenetic modification that is both responsive to environmental factors, and

48   associated with gene regulation and phenotype (Klose & Bird, 2006; E. Li & Bird, 2007). When

49   considering the potential biological impact of DNA methylation, a key consideration is that its

50   biological function (e.g. permissive vs. repressive effects on gene expression) depend in large

51   part on where DNA methylation occurs within the genome – i.e., in repetitive elements, gene

52   promoter regions, or gene bodies (Jones, 2012; Schübeler, 2015).

53   Of particular interest in this paper is DNA methylation of CpG sites in CCGG motifs

54   located throughout the mammalian genome. In a cross-species comparison, we identified 2.19

55   million CCGG motifs in the dog canFam3 genome assembly (Lindblad-Toh et al., 2005), 2.75

56   million CCGG motifs in the cat felCat8 genome assembly (Lindblad-Toh et al., 2011), and 2.46

57   million CCGG motifs in the human hg38 genome assembly (Lander et al., 2001). Approximately

58   97% of CCGG motifs in the human genome occur in gene bodies and repetitive sequence

59   regions of DNA, away from transcription start sites (Ball et al., 2009; Kinney et al., 2011). Taken

60   together, there appears to be broad conservation of the CCGG motif across mammalian taxa

61   and this motif is apparently distributed throughout the genome. At this scale, we and others

62   (Vryer & Saffery, 2017) refer to this metric as global DNA methylation, as it is a composite

63   average of methylation sampled from CpG sites ubiquitously dispersed across the genome.

64   Global DNA methylation is distinct from "genome-wide DNA methylation," which refers to DNA

65   methylation measured across the genome at region-specific or single-nucleotide resolution. In

66   general, global DNA methylation is thought to be an indicator of genomic stability (Schulz, 2006;

67   Slotkin & Martienssen, 2007), as genome-wide hypomethylation is associated with high

68   mutation rates and human cancers (Chen, Pettersson, Beard, Jackson-Grusby, & Jaenisch, 1998;

69   Feinberg & Vogelstein, 1983; Woo & Kim, 2012).

70   Beyond its relevance to health outcomes, global DNA methylation has also garnered

71   interest as a biomarker of environmental exposures, thereby serving as a potential pathway

72   linking experiences to phenotype. In humans, the nutritional environment and socioeconomic

73   circumstance during early life, namely gestation (Boeke et al., 2012) and early childhood (Perng

74   et al., 2012), are associated with global DNA methylation measured at LINE-1 repetitive

75   elements. In rodents, maternal treatment with a synthetic stress hormone (betamethasone)

76   causes a decrease in offspring global DNA methylation (Crudo et al., 2012), suggesting that

77   social stressors that increase natural glucocorticoid level might also affect global DNA

78   methylation. Rodent models also provide evidence that maternal nutritional supplementation is

79   associated with global DNA methylation measured in offspring fetal tissues (Kovacheva et al.,

80   2007; Kulkarni et al., 2011).

81   Despite the numerous human and rodent studies assessing DNA methylation within the

82   context of the DOHaD hypothesis, there is a need to combine approaches used in biomedical

83   research with research done in wild animals (Lea, Tung, Archie, & Alberts, 2017). Integration of

84   DOHaD concepts (e.g., life course biology) with molecular data (e.g., DNA methylation) is

85   especially salient in long-lived gregarious species. Such efforts in wild animals could better

86   enable investigators to explore not only how naturally-occurring environmental factors might

87   affect DNA methylation, but also, the extent to which variation in DNA methylation patterns is

88   detectable across the life span (Laubach et al., 2018). These efforts will ultimately pave the road

89   for studies evaluating the relationships among environmental factors, DNA methylation,

90   phenotype, and fitness, which are relevant in an evolutionary context given that variations in

91   phenotype and health are shaped by natural selection (Laubach et al., 2018).

92   **Objectives and hypotheses**

93   In the present study, we test the hypothesis that early life social and ecological factors

94   are determinants of global DNA methylation (%CCGG methylation) in three key age classes

95   (cub, subadult, and adult) in a population of wild, spotted hyenas (*Crocuta crocuta*). For the

96   early life social/ecological factors, we focus primarily on the social rank of each individual

97   hyena's mother during the year in which it was born as our primary explanatory variable of

98   interest ("maternal rank"). This rationale stems from the fact that social rank is a known

99   determinant of priority of access to resources (Frank, 1986; Holekamp, Smith, Strelioff, Van

100 Horn, & Watts, 2012; Tilson & Hamilton, 1984) and fitness (Höner et al., 2010; Swanson,

101 Dworkin, & Holekamp, 2011) in spotted hyenas. In addition, we also consider litter size, extent

102 of anthropogenic disturbance during the hyena's birth year, and prey availability. We predicted

103 positive associations of both maternal rank and prey density during early life with global DNA

104 methylation. We also predicted that larger litter size and more exposure to human disturbance

105 during the hyena's birth year would be associated with lower global DNA methylation. For all

106 relationships of interest, we anticipated larger magnitude of associations during earlier than

107 later age classes given that explanatory variables were measured during a hyena's birth year.

108

109 **METHODS**

110 **Study population**

111   We used samples and data collected by personnel from the Mara Hyena Project, a long-

112 term field study of wild spotted hyenas in the Masai Mara National Reserve, Kenya. Spotted

113 hyenas are gregarious carnivores that live in large groups known as clans (Kruuk, 1972). Within

114 each clan, relationships among individuals are structured by a linear dominance hierarchy

115 organized by matrilines, and a cercopithecine primate-like pattern of youngest ascendency

116 during the process of rank acquisition (Engh, Esch, Smale, & Holekamp, 2000; Holekamp &

117 Smale, 1991; Holekamp & Smale, 1993; Smale, Frank, & Holekamp, 1993). A hyena's rank

118 determines not only its priority of access to such critical resources as food and mates, but also

119 the nature of its social interactions with other clan members; both resource access and social

120 interaction patterns are known to affect fitness in this species (Frank, 1986; Holekamp et al.,

121 2012; Holekamp, Smale, & Szykman, 1996; Smith, Memenis, & Holekamp, 2007). Female

122 hyenas typically give birth to 1-2 offspring (Frank, Glickman, & Licht, 1991; Holekamp et al.,

123 1996), which depend on their mothers for food and protection until offspring are approximately

124 two years of age (Watts, Tanner, Lundrigan, & Holekamp, 2009). The importance of social

125 status in hyena societies, and the protracted period of maternal dependence, make this species

126 a good model system in which to test our hypothesis.

127   Demographic, behavioral, and biological sample data have been collected continuously

128 since 1988 from individual hyenas identifiable by their unique spot patterns. For the present

129 analysis, we selected a subset of 381 hyenas for which we have both detailed behavioral data

130 for calculation of maternal rank (the primary explanatory variable of interest) and archived

131 blood samples for quantification of global DNA methylation (the dependent variable of

132 interest). After completing Quality Assessment and Quality Control (QA/QC) of DNA

133 methylation values, our final analytic sample comprised 293 individual hyenas belonging to six

134 clans (see **Supplemental Material**). Of these individuals, 58 had repeated measures capturing

135 more than one age class due to the opportunistic nature of immobilizations and blood draws.

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

137   *Early life social environment*

138   *Maternal rank*.

139   We determined the social rank of each adult female based on her wins and losses in

140   dyadic agonistic interactions (Engh et al., 2000; Holekamp & Smale, 1993; Smale et al., 1993).

141   Each individual's rank was updated annually. To characterize the early life social environment,

142   each cub was assigned the rank held by its mother, called its maternal rank, during the year in

143   which it was born. In order to account for differences in clan size and yearly demographic

144   changes, we standardize rank on a relative scale from -1, corresponding to the lowest ranking

145   adult female, to 1, corresponding to the highest-ranking female.

146   *Litter size.*

147   In addition to interacting with its mother, each young hyena also interacts and

148   competes with its littermate, if it has one (Frank et al., 1991; Holekamp et al., 1996). Based on

149   daily observations of our study clans, we determined whether each hyena belonged to

150   singleton or twin litter when it was first seen above ground.

151   *Ecological factors*

152   *Anthropogenic disturbance during the birth year.*

153   We categorized anthropogenic disturbance based on the amount of illegal livestock

154   grazing in the Reserve by pastoralist Masai herdsman under two different management

155   regimes. Based on data collected and analyzed by Green et al. (2018), we assigned hyenas in

156   each clan in each year to one of three categories of human disturbance: high, medium, and low

157   (Green, Johnson-Ulrich, Couraud, & Holekamp, 2018). Levels of human disturbance were based

158   on livestock counts that began in 2000 in the eastern part of the reserve near the Masai town

159   of Talek. The counts were conducted systematically throughout the year and the total number

160   of livestock counted were averaged annually. Livestock were never observed on the western

161   side of the reserve, and illegal grazing did not proliferate near Talek until around 2000 (personal

162   observation).

163   *Prey density during discrete developmental periods.*

164   Twice each month, research assistants counted all prey animals observed within 100

165   meters of either side of established 4-km prey transect routes in the territories of our study

166   clans. Details of these methods are presented elsewhere (Cooper, Holekamp, & Smale, 1999;

167   Green et al., 2018). We combined counts of impala (*Aepyceros melampus*), plains zebra (*Equus*

168   *burchelli*), Thomson's gazelle (*Eudorcas thomsonii*), topi (*Damaliscus lunatus*), and white-

169   bearded wildebeest (*Connochaetes taurinus*), which are the primary wild ungulate prey of

170   hyenas in the Reserve, comprising at least 93% of the prey hunted by hyenas there (Holekamp,

171   Smale, Berg, & Cooper, 1997). We estimated the average prey density during five discrete 3-

172   month periods in the hyena's early life so that we could identify sensitive periods for exposure

173   to varying nutritional regimes. For each hyena from our study population, we calculated the

174   average number of ungulate prey during the peri-conceptional period (1.5 months before and

175   1.5 months after conception), during gestation (3 months prior to birth), from birth to 3

176   months, 3-6 months, and 6-9 months. These five periods were selected because they cover key

177   developmental periods, starting with their mother's access to food before conception, covering

178   the 110-day gestation period, and extending through early post-natal ontogeny (Holekamp &

179   Smale, 1998; Kruuk, 1972). Our approach to modeling associations of food availability at

180   discrete time periods during gestation and early life with later life phenotypes was intended to

181   parallel an analytical approach used by researchers studying the Dutch Hunger Famine (Painter,

182   Roseboom, & Bleker, 2005).

183   *Life history traits*

184   *Sex.*

185   We determined the sex of each hyena based on the glans morphology of its erect

186   phallus during field observations; this is reliable starting at 3 months of age (Frank, Glickman, &

187   Powch, 1990).

188   *Age.*

189   We aged hyenas by back-calculating their birthdates based on their physical appearance

190   when first observed as infants. Based on their pelage, morphology and behavior, we are able to

191   determine a cub's age with an accuracy of ± 7 days (Holekamp et al., 1996). We used this

192   method to determine each hyena's age in months at the time of blood collection. Because we

193   were interested in associations of birthyear socioecological factors on DNA methylation among

194   different age classes of hyenas, we also operationalized age at blood collection as a 3-level

195   variable – cub, subadult, and adult – corresponding to prominent life-history milestones during

196   development. We defined the cub age class as ≤12 months of age (Holekamp & Smale, 1998),

197   which approximately coincides with the mean age of weaning (11.9 months) in this subsample

198   of our study animals. The subadult age class was defined as >12 to ≤24 months of age. The

199   adult age class was classified as >24 months of age, as hyenas become reproductively

200   competent at 24 months (Holekamp & Smale, 1998; Holekamp et al., 1996).

201   **Dependent variable: global DNA methylation**

202   *Blood collection and DNA extraction.*

203   Hyenas were immobilized using 6.5 mg/kg of tiletamine-zolazepam (Telazol ®) delivered

204   in a pressurized dart fired from a CO_2 powered rifle (Telinject Inc.). We collected blood from the

205   hyena's jugular vein into ethylenediaminetetraacetic acid (EDTA) coated vacuum tubes. The

206   samples were flash frozen in liquid nitrogen or processed for genomic DNA extraction (using the

207   Gentra Pure Gene kit by Qiagen®) then stored in -80°C freezers until time of analyses.

208   At the time that we selected blood samples for DNA methylation assays, we noted the

209   date of sample collection and calculated sample age, which was included in a sensitivity analysis

210   to assess whether or not there was potential variation in DNA quality due to storage time.

211   *Global DNA methylation assay.*

212   We quantified global DNA methylation as percent methylated CCGG sites (%CCGG

213   methylation) in peripheral leukocytes using LUMA (Karimi, Johansson, & Ekström, 2006; Karimi

214   et al., 2006). Extensive details on our laboratory methods and QA/QC are included in the

215   **Supplemental Material**. Briefly, this method uses both methyl sensitive (*HpaII*) and methyl

216   insensitive (*MspI*) restriction enzymes that target a shared recognition motif of CCGG

217   throughout the genome. In mammals, there are roughly 2.4 million CpG sites at CCGG motifs.

218   Generalizing among mammals by using the well-annotated human genome, approximately 3%

219   of CpG sites belonging to the CCGG motif are near (< 1kb) transcription start sites, 45% are in

220   gene bodies, and 52% are in non-coding repetitive elements (Ball et al., 2009; Kinney et al.,

221   2011). Given the high proportion of CpG sites within gene bodies and non-coding repetitive

222   elements, we suspect that higher %CCGG methylation measured via the LUMA assay may

223   reflect regulation of transcription and alternative splicing (Lev Maor, Yearim, & Ast, 2015; Li,

224   Zhang, Huang, & He, 2018) as well as repression of repetitive elements (Barau et al., 2016;

225   Coluccio et al., 2018) and enhanced chromosomal stability (Eden, Gaudet, Waghmare, &

226   Jaenisch, 2003; Tuck-Muller et al., 2000). Accordingly, we cautiously interpret higher %CCGG

227   methylation as a more favorable outcome than lower %CCGG methylation.

228   **Statistical analyses**

229   Prior to formal analysis, we performed a series of quality control assessments and

230   evaluation on our data. First, we examined the distribution of continuous variables (%CCGG

231   methylation, prey density, age in months), and assessed frequency of nominal categorical

232   variables (sex, maternal rank quartiles, litter size [singleton vs. twin], human disturbance during

233   birth year [low, medium, high]) for deviations from normality, and to identify missing values.

234   Next, given the potential impact of shared genes among siblings on DNA methylation (Hannon

235   et al., 2018), we calculated intraclass correlations (ICC) comparing within and between family

236   variability in %CCGG methylation based on the premise that an ICC >0.1 indicates greater within

237   than between family correlation (i.e., lower within than between family variability) which would

238   warrant a need to account for shared genes in the analysis. Third, because sex (Doherty et al.,

239   2016) and age (Bjornsson et al., 2008) can potentially alter the relationship between early

240   exposures and DNA methylation, we assessed for effect modification by sex and by age on the

241   relationship between maternal rank (our primary explanatory variable of interest) and %CCGG

242   methylation using linear mixed models. We accounted for the repeated measurements of DNA

243   methylation from the 58 individuals with more than one DNA methylation value by including a

244   random intercept for hyena ID. Here, we considered stratified analysis if the P-value for the

245   interaction term was <0.20. The tests for interaction indicated effect modification of the

246   relationship between maternal rank and offspring %CCGG methylation by age group, so we

247   carried out subsequent analyses separately for cub, subadult, and adult hyenas. Finally, we

248   examined bivariate associations between the explanatory variables and %CCGG methylation

249   among all hyenas in the study. We conducted bivariate analysis using a linear mixed model with

250   a random effect for individual identity (to account for repeated measurements) to explore

251   crude associations between our explanatory variables and %CCGG.

252   For the main analysis, we examined associations between each explanatory variable and

253   %CCGG methylation separately for cubs (n = 65), subadults (n = 127), and adults (n = 127). We

254   employed this analytical strategy to explore the extent to which early life environment was

255   associated with DNA methylation at different stages of development. We acknowledge that we

256   have three cross-sectional populations rather than one longitudinal population due to

257   constraints on available archived samples. In the analysis, we used linear regression models to

258   examine unadjusted and adjusted associations between each explanatory variable and %CCGG

259   methylation within each life-stage category. In the adjusted models, we explored the extent to

260   which each of the explanatory variables was associated with %CCGG methylation after

261   controlling for key covariates, including a hyena's continuous age in months at the time of

262   darting and sex (Model 1). We assessed residual plots for each multiple variable regression

263   model and conducted a Breusch-Pagan test to check for violations of homoskedasticity.

264   In adults only, we ran an additional model in which maternal rank was the explanatory

265   variable of interest and %CCGG methylation was the continuous outcome. This model, which

266   was limited to adult females, included continuous age in months as a covariate and each

267   hyena's own rank during the year in which it was darted. Inclusion of the hyena's own rank

268   allowed us to assess the independent effects of maternal rank after hyenas had taken their

269   places in the rank hierarchy.

270   In models where prey density was the explanatory variable of interest, we also

271   controlled for prey density during all previous developmental periods to isolate the

272   independent effect of the period of interest. That is, we treated earlier prey density as a

273   confounding variable that, if not controlled for in our model, could bias our estimate of

274   association for current prey density and offspring DNA methylation.

275   Finally, based on results of our adjusted Model 1 for each age group, we implemented

276   Model 2, which mutually adjusted for statistically significant (*P*<0.05) explanatory variables

277   from Model 1. That is, Model 2 included all of the covariates (e.g. sex and age in months) in

278   addition to all explanatory variables that were significantly associated in Model 1 with %CCGG

279   methylation. By doing this, Model 2 enabled us to document the independent effects of the

280   strongest determinants of %CCGG methylation.

281   *Sensitivity analyses*

282   In sensitivity analyses, we evaluated the potential impact of sample storage time on

283   DNA methylation measurement by additionally including the year during which DNA was

284   extracted and put into our freezer as a covariate in the models. In addition to comparing the

285   direction, magnitude, and precision of the estimates, we also calculated the variance inflation

286   factor (VIF) to test for collinearity among covariates given that both sample age and

287   anthropogenic disturbance are both based on the time order of years during the project.

288

289   **RESULTS**

290   **Descriptive statistics**

291   Slightly more than half the study population were females (56%), and we had more

292   samples from individuals at older than young life-stages; 20% were cubs, 40% were subadults,

293   and 40 % were adults. Most sampled individuals (79%) were members of twin litters and 21%

294   were singletons. Additional sample characteristics are shown in **Table 1**.

295   Our indicator of global DNA methylation, %CCGG methylation in peripheral leukocytes,

296   was relatively normally distributed with a mean ± SD of 75.75 ± 2.79% (**Figure 1**). In bivariate

297   analysis, there was no significant difference in %CCGG methylation between male and female

298   hyenas (males 75.57 ± 3.09 and females 75.89 ± 2.55 %CCGG methylation; difference = -0.30

299   [95% CI: -0.93, 0.33], P-value = 0.35). We noted a positive monotonic relationship between

300   hyena age category and %CCGG methylation: 74.90 ± 3.64% in cubs, 75.82 ± 2.60% in subadults,

301   and 76.12 ± 2.38% in adults (F-statistic = 4.58, P-value = 0.02).

302   **Data checks**

303   We found no evidence of familial clustering, with ICCs of 0.052, 0.077, and 0.000 in

304   cubs, subadults, and adults, respectively. Given that an ICC >0.1 is considered the cut-off for the

305   need to cluster by a variable (Vajargah & Masoomehnikbakht, 2015), these low ICCs suggest

306   that familial clustering is not an issue in our data, and thus, not accounted for in the models.

307   We also tested for a statistical interaction between sex and age class (cub, subadult, and

308   adult) with maternal rank (our primary explanatory variable of interest) on %CCGG methylation,

309   which revealed evidence of effect modification with age (*P*-interaction = 0.06) but not sex (*P*-

310   interaction = 0.42). Given the effect modification with age, in addition to our *a priori* interest in

311   investigating the extent to which associations between early experiences and DNA methylation

312   are observed across development, we stratified all subsequent analyses by age class. Because

313   the relationship between maternal rank and %CCGG methylation in cubs was not monotonic

314   (**Figure 2**), we binned standardized maternal rank into quartiles, with the first quartile

315   representing lowest maternal rank and the fourth quartile representing highest maternal rank.

316   Prior to our age stratified analyses, we also ran a model in which %CCGG methylation is the

317   dependent variable, and explanatory variables included: offspring sex, maternal rank, offspring

318   age groups (cub, subadult, and adult) and a maternal rank*offspring age group interaction

319   term. While the beta estimates from the interaction model are more limited in their

320   interpretation than the stratified models discussed below, there was concordance between

321   these results (**Supplemental Table 1**).

322   **Cub Models**

323   **Table 2** shows the Model 1 adjusted associations between explanatory variables and

324   %CCGG methylation in hyenas during the cub life-stage (for unadjusted estimates, see

325   **Supplemental Table 2**). In Model 1, which accounted for the hyena's age (in months) and sex,

326   maternal rank was positively associated with %CCGG methylation. Specifically, hyena cubs

327   whose mothers were in the second, third, and fourth quartiles of rank had 3.19 (95%CI: 0.68,

328   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)

329   higher % CCGG methylation, respectively, than those whose mothers were in the lowest rank

330   quartile. The relationship between maternal rank and %CCGG methylation was positive but not

331 strictly monotonic. We also found that, compared to cubs born into low anthropogenic

332 disturbance, cubs from medium anthropogenic disturbance groups had 2.88 (95%CI: 0.99, 4.78;

333 P = 0.004) %CCGG higher methylation and cubs born into high disturbance had 3.51 (95%CI:

334 0.83, 6.19; P = 0.013) %CCGG higher methylation. On the other hand, density of wild ungulate

335 prey periconceptionally and from birth to 3 months of age was inversely related to %CCGG

336 methylation. In unadjusted analysis, we found that for every 1 SD of wild ungulate prey density

337 measured periconceptionally there was 1.24 (95%CI: 0.43, 2.04; P = 0.004) lower %CCGG

338 methylation, and each 1 SD of wild ungulate prey density measured from birth to 3 months

339 corresponded to 1.55 (95% CI: 0.53, 2.58; P = 0.004) lower %CCGG methylation. Adjusting for

340 hyena's age, sex, and previous prey period densities in Model 1 slightly attenuated the

341 associations at periconception (-1.18 [95% CI: -2.03, -0.33]; P = 0.009), and from birth to 3

342 months (-1.40 [95% CI: -2.44, -0.36]; P = 0.011). In Model 2, we mutually adjusted each

343 significant explanatory variable from our previous models (Model 1) by including maternal rank,

344 anthropogenic disturbance, and average wild ungulate prey density from periconceptional,

345 gestational, and birth to 3 months as fixed effects parameters in the same model. Doing so

346 attenuated estimates for anthropogenic disturbance and prey density from periconception to 3

347 months but not for maternal rank (and in fact, slightly strengthened the associations involving

348 maternal rank), nor did it substantially widen the confidence intervals for maternal rank or wild

349 ungulate prey density (**Figure 3**).

350 **Subadult models**

351   **Table 2** also shows results for subadult hyenas. We did not observe any statistically

352 significant associations between maternal rank and %CCGG methylation in this age group after

353 adjusting for covariates in Model 1. As with our cub models, we found that medium and high

354 anthropogenic disturbance corresponded to 0.84 (95% CI: -0.44, 2.13; P = 0.201) and 2.05 (95%

355 CI: 0.85, 3.25; P = 0.001) higher %CCGG methylation, respectively. We also noted a trend

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

357 litters.

358 **Adult models**

359   In the last column of **Table 2**, we show associations for adult hyenas. In Model 1, we

360   again observed no effect of maternal rank on %CCGG methylation. In a subset of adult females,

361   we ran an additional model that controlled for each hyena's own rank and similarly found no

362   significant effect of maternal rank on %CCGG methylation **(Supplemental Table 3)**. However,

363   there was an inverse association between wild ungulate prey density from birth to 3 months of

364   age and %CCGG methylation. Each 1 SD increment in prey density corresponded with 0.68 (95%

365   CI: 0.22, 1.14; P = 0.005) lower % CCGG methylation. None of the other early life social or

366   ecological variables were related to global DNA methylation in this age group.

367   **Sensitivity analyses**

368   Results from models where we further adjusted for sample age as a covariate were

369   similar to those without sample age adjustment (**Supplemental Table 4**). These findings, in

370   conjunction with potential collinearity between sample age and some of the explanatory

371   variables of interest (particularly in models for anthropogenic disturbance which had VIFs > 5.0)

372   and a recent publication demonstrating the robustness of DNA methylation to storage time (Y.

373   Li et al., 2018), serve as the impetus for us to focus the discussion of results on models that do

374   not include sample age.

375

376   **DISCUSSION**

377   In this study of 293 wild cub, subadult and adult spotted hyenas in Kenya, we sought to

378   identify early life social and ecological explanatory variables of global DNA methylation, as

379   indicated by % methylated CCGG across the genome. In line with our expectations, we found

380   that higher maternal rank at birth was associated with higher global DNA methylation in

381   offspring sampled as cubs, but not in those sampled as subadults or adults. Among cubs and

382   subadults, higher anthropogenic disturbance during the year in which hyenas were born

383   corresponded to greater methylation. We also found an unexpected inverse relationship

384   between prey density (an indicator of food availability) measured during the peri-conceptional

385   period through the first three months of life and global DNA methylation in offspring sampled

386   as cubs and adults. Associations in cubs were robust to mutual adjustment, suggesting

387   independent effects of perinatal social environment and food availability on later life global

388   DNA methylation.

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

390   A comparison of %CCGG methylation of DNA extracted from whole blood using the

391   LUMA assay shows that hyenas have similar global methylation to other vertebrates, including

392   both another member of the order Carnivora and humans. For example we observed only 0.7%

393   less methylation in hyenas than domestic dogs (Montrose et al., 2015), and hyenas had

394   approximately 3.7% greater methylation than humans (Ono et al., 2012; Virani et al., 2012).

395   **Maternal rank and global DNA methylation**

396   Our most notable finding was a positive, albeit not strictly monotonic, relationship

397   between maternal rank and global DNA methylation in cubs. Specifically, we found that cubs

398   born to mothers in the upper three rank quartiles had 2-3% higher CCGG methylation than

399   those whose mothers were in the lowest rank quartile. This association may reflect the fact that

400   offspring of high-ranking mothers have greater access to social capital and resources, which in

401   other gregarious species, predict positive health outcomes (Sapolsky, 2005). Work on rhesus

402   macaques (*Macaca mulatta*) revealed differences in DNA methylation at more than 25,000

403   genomic locations in placental tissue when comparing offspring from high- and mid-rank

404   mothers to those of low-ranking mothers (Massart et al., 2017). Similarly, differential DNA

405   methylation across the genome was also observed in a recent human study that reported

406   associations between socioeconomic status (SES) and DNA methylation at nearly 500 CpG sites

407   in young children (Bush et al., 2018). Using a more focused candidate gene approach, three

408   studies of humans quantified methylation of genes involved in growth (King, Murphy, & Hoyo,

409   2015; Obermann-Borst et al., 2012) and regulation of stress hormones (Appleton et al., 2013) in

410   cord and infant blood, and found variation in gene-specific DNA methylation with maternal

411   education and household income, which are both strong indicators of SES. Taken together,

412   these studies point toward an effect of early life social status on DNA methylation patterns that

413   is detectable as early as the day of birth. Of particular relevance to the present study are

414   findings from school-age children that higher family SES level was associated with higher global

415   DNA methylation (LINE-1 repetitive element) in boys (Perng et al., 2012). These findings are

416   pertinent to our results given not only similarities in the types of independent (social status)

417   and dependent variables (global DNA methylation) of interest, but also considering that both

418   studies assessed social status early in life and metrics of global DNA methylation in post-natal

419   juveniles.

420   We did not observe any relationship between maternal rank and %CCGG methylation

421   among subadult or adult hyenas, even after controlling for adult hyenas' own ranks. There are a

422   few potential explanations for the null findings in later life-stages. First, the epigenome is labile

423   and responsive to the environment across ontogeny. In this particular study, %CCGG

424   methylation, presumably established in association with maternal rank at birth, may be further

425   modified in response to a hyena's own rank and related social or ecological factors, especially

426   during later life-stages when the hyena becomes less dependent on its mother. Studies in

427   rhesus macaques (Tung et al., 2012) and humans (Mcguinness et al., 2012) have reported

428   marked variation in genome-wide and global DNA methylation in adulthood with respect to

429   current social rank and SES, respectively, suggesting potential effects of one's current social

430   environment on the epigenome. Similarly, Subramanyam et al. found no relationship between

431   early life SES and adult global DNA methylation (LINE-1 and ALU repetitive elements) in 998

432   participants of a large multi-ethnic population of middle-aged adults in the U.S. However, the

433   authors did find that attained wealth, a socioeconomic asset accrued across the life span, was

434   associated with higher methylation of both LINE-1 and ALU (Subramanyam et al., 2013). A

435   subsequent study of the same subject population investigated effects of early life and adult SES

436   on gene-specific methylation, and found that SES at both time-points was associated with

437   differential methylation – with both positive and negative directions of associations – of specific

438   genes in adulthood, although the subset of genes affected by childhood and adult SES did not

439   completely overlap (Needham et al., 2015). Together these findings suggest that, although

440   social status clearly affects the epigenome, these effects likely vary not only across different life

441   stages, but also, with respect to detectable differences in DNA methylation assessed at specific

442   loci vs. at the global level. Further, effects of SES that are apparent early in life may not persist

443   throughout ontogeny. In the present study, our *a priori* hypothesis focused on the effect of the

444   early life environment on later DNA methylation. However, we did consider the potential

445   effects of a hyena's own rank given that this might contribute to the null associations that we

446   observed during later life-stages. Nevertheless, controlling for an adult hyena's own rank did

447   not reveal a significant effect of maternal rank on %CCGG methylation in adults.

448   Another potential explanation for the null findings in subadults and adults revolves

449   around the fact that recapitulation of DNA methylation patterns is not perfect. That is, DNA

450   methylation may change over time due to random errors. A longer time elapsed from original

451   establishment of DNA methylation patterns *in utero* corresponds to greater potential for errors

452   to occur in DNA methylation replication mechanisms (Laubach et al., 2018).

453   **Anthropogenic disturbance**

454   Mid- and high-level human disturbance, based on the year in which a hyena was born,

455   were positively associated with cub and subadult global DNA methylation. Although we

456   expected that human disturbance would be negatively associated with global DNA methylation,

457   we observed a positive anthropogenic disturbance effect that appeared to be strongest among

458   cubs and was evident in subadults. Regardless, these findings are interesting from a biological

459   viewpoint given that hyenas are generalist hunters that thrive under medium disturbance

460   (Cooper et al., 1999; Green et al., 2018). It may be that higher levels of anthropogenic activity

461   enhance availability of livestock as prey for local hyenas. We know that Masai livestock are

462   utilized as a food source by our study animals when they are available as potential prey (Green

463   et al., 2018; Kolowski & Holekamp, 2006), and this nutritional abundance may be reflected in

464   the epigenome.

465   **Prey density**

466   We observed an inverse association between prey density in the first three months of

467   life and global DNA methylation in cubs and adults. This is the opposite of what we had

468   hypothesized, given that dietary intake of methyl-donor nutrients provides the primary

469   substrate for the DNA methylation reaction (Anderson, Sant, & Dolinoy, 2012). Although these

470   results were unexpected, one potential explanation may involve increased social stress during

471 periods of food abundance. In our study population, we have repeatedly noted an increase in

472 the rate at which hyenas engage in social interactions (both positive or negative) during periods

473 of greater prey abundance (eg., Holekamp et al., 2012). Furthermore, we found that fecal

474 glucocorticoid levels are elevated during periods of higher prey abundance among juvenile but

475 not pregnant adult female hyenas (Greenberg, 2017). Although greater prey abundance was

476 not associated with higher stress levels in pregnant females in this analysis, it is possible that

477 we were underpowered to detect an effect among pregnant females given that we had

478 measurements from only 31 of them compared to 123 juvenile hyenas. Given this caveat,

479 elevated stress hormones are known to be associated with DNA methylation. For example, an

480 experimental study of guinea pigs revealed that *in utero* exposure to elevated glucocorticoid

481 levels caused lower global DNA methylation assessed via LUMA (Crudo et al., 2012). If a more

482 powerful analysis reveals that pregnant female hyenas have elevated glucocorticoids during

483 higher than lower prey abundance periods, then this could potentially explain the inverse

484 association we observed between prey abundance and global DNA methylation. Second, in

485 contrast to many other hyena populations in Africa, food is very seldom in short supply for

486 Mara hyenas such that periods of low prey abundance experienced by this population do not

487 induce nutritional stress in hyenas, at least not comparable to famine exposed humans

488 (Heijmans et al., 2008; Tobi et al., 2009).

489 **Litter size**

490 Besides the above-mentioned findings, there is another association worth noting. We

491 observed that twins had lower global DNA methylation than singletons during the subadult life-

492 stage, although this association was not statistically significant. That this effect is only observed

493 in subadults makes sense in light of the fact that either social or nutritional stress from

494 competition with a sibling may accumulate during the months prior to weaning and during the

495 subadult life stage.

496 **Strengths and limitations**

497 Our study had a number of strengths, including its large sample size, the use of a novel,

498 long-lived social mammal as a model organism, and the availability of rich meta-data on

499   demographic, behavioral, and ecological factors that might influence DNA methylation. These

500   unique data coupled with biological samples collected from hyenas at different life stages

501   allowed us to test DOHaD hypotheses in a wild animal system.

502   Our study also has clear limitations. First, we used the LUMA assay, which is a reliable

503   and a particularly attractive option for wild animals lacking well curated genomes (Head, Mittal,

504   & Basu, 2014). However, the CCGG sites targeted by this assay represent a single composite

505   average of genomic DNA methylation and do not provide any information on finer resolution

506   differences in DNA methylation that may be relevant to environmental risk factors and/or

507   phenotypes. For example in humans, Waterland et al. (2010) found that individuals who were

508   conceived during seasonal food shortages exhibited higher DNA methylation at metastable

509   epialleles but no differences in global measures of DNA methylation (LINE-1) or DNA

510   methylation of imprinted genes during childhood. Future studies using genome-wide

511   approaches, such as Reduced Representation Bisulfite Sequencing (Meissner et al., 2005), are

512   warranted to home in on specific regions of the genome that may demonstrate changes in DNA

513   methylation related to the environment and/or phenotypes.

514   Another limitation is our use of archived DNA extracted from blood but without

515   information on cellular heterogeneity (i.e., proportion neutrophils, eosinophils, basophils,

516   lymphocytes, and monocytes), which may be relevant given that there is cell type-specific

517   variation in DNA methylation (Adalsteinsson et al., 2012). However, we believe our measure of

518   global DNA methylation, taken as an average across leukocyte cell types, is still valuable given

519   that environmental exposures like social stress (Engler, Bailey, Engler, & Sheridan, 2004) and

520   infection (Helmby, Jönsson, & Troye-Blomberg, 2000) affect the cellular composition and the

521   distribution of leukocyte subpopulations. Therefore, the "effects" of our explanatory variables

522   on DNA methylation may well include their effects on cell type composition.

523   Other limitations include 1) the fact that our study design is cross-sectional (which does

524   not allow assessment of within-individual change over time, and is generally prone to suffer

525   from reverse causation and unmeasured confounding (Greally, 2018; Lappalainen & Greally,

526   2017)); 2) the potential for sample selection bias (e.g., offspring from low ranking lineages,

527   which presumably have lower DNA methylation, are in worse condition and may be less likely

528   to survive to older ages thus reducing variation in DNA methylation in the older age classes);

529   and 3) a possible time-varying effect of our explanatory variables on the epigenome throughout

530   a hyena's life, thus limiting the extent to which we can identify causal relationships from data

531   collected at specific time-points (Mansournia, Etminan, Danaei, Kaufman, & Collins, 2017).

532   Finally, we cannot discount the possibility of chance findings given the number of

533   models tested. However, our research focus was to describe and assess the direction,

534   magnitude, and precision of the estimates rather than focus on statistical significance,

535   especially in light of the fact that our explanatory variables were related biological concepts

536   and included correlated variables, like prey density during successive time periods. In such

537   scenarios, use of multiple comparisons corrections would unfairly penalize models containing

538   correlated explanatory variables of interest and increase risk of type 2 error at the cost of

539   reducing type 1 error (Rothman, 1990).

540

541   **Conclusions**

542   In conclusion, we found that maternal social rank at the time of birth was positively

543   associated with %CCGG methylation in hyena cubs, but not in subadult or adult hyenas. We also

544   found that higher anthropogenic disturbance at birth, which is possibly an indicator of a reliable

545   and easy to catch food source (i.e. domestic livestock) corresponded with higher global DNA

546   methylation in cubs and subadults. Finally, availability of wild ungulate prey at periconception

547   (among cubs only) and from birth to 3-months of age was related to lower global DNA

548   methylation in cub and adult hyenas, a finding that requires further investigation and testing of

549   alternative hypotheses regarding the role of social stress.

550   Given that %CCGG DNA methylation represents coverage in gene bodies and noncoding

551   repetitive sequences of DNA (Ball et al., 2009; Kinney et al., 2011), and that higher methylation

552   of these regions is associated with intragenic exon expression (Li et al., 2018), lower rates of

553   transposon activity (Barau et al., 2016) and genomic stability (Eden et al., 2003; Tuck-Muller et

554   al., 2000), our findings suggest that social and ecological experiences during early life that are

555 associated with lower global DNA methylation may also be determinants of adverse

556 phenotypes or lower fitness in hyenas – a topic for future studies. Furthermore, we recommend

557 longitudinal studies to directly assess the persistence of epigenetic modification over

558 ontogenetic development in long-lived and gregarious species. Finally, incorporating additional

559 information on early life social experience (e.g., maternal care and interactions with peers)

560 should reveal novel insights into how social interactions shape the epigenome (Massart et al.,

561 2017; Provencal et al., 2012; Weaver et al., 2004) in the context of DOHaD.

562

575

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884 **DATA ACCESSIBILITY:**

885 Data, including independent variables and LUMA DNA methylation values will be archived on

886 GitHub at, https://github.com/laubach/hy_luma. The R analysis code is also stored here and

887 available for public access.

888

889 **AUTHOR CONTRIBUTIONS**

890 Z.M.L. conceived the research, did the laboratory work and statistical analyses, and wrote the

891 manuscript. K.E.H., D.C.D. and C.D.F. provided financial and intellectual support, helped refine

892 hypotheses and experimental designs, and provided oversight on interpretation of results. L.M.,

893 T.R.J., and D.R. assisted with laboratory work and provided feedback on the manuscript. M.O.P.

894 darted hyenas and collected field data.

Tables and Figures

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

	$N^{\dagger}$	%
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%

	N	Mean ± SD

Average prey density during discrete developmental periods (per 1 km²)[§]

	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 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[†]	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, 0.20)	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 1 Life history and social characteristics of 293 spotted hyenas as well as ecological measures from the Masai Mara, Kenya.

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Singleton	44	21%
Twins	164	79%
ECOLOGICAL CHARACTERISTICS		
Anthropogenic disturbance during birth year[‡]		
Low	102	36%
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Average prey density during discrete developmental periods (per 1 km^2)[§]	N [†]	Mean ± SD
Periconception	237	237.3 ± 162.6
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[†] 320 measurements from 293 individual hyenas; Ns may not add up to 293 individuals, due to missing values.

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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, 0.20)	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

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.
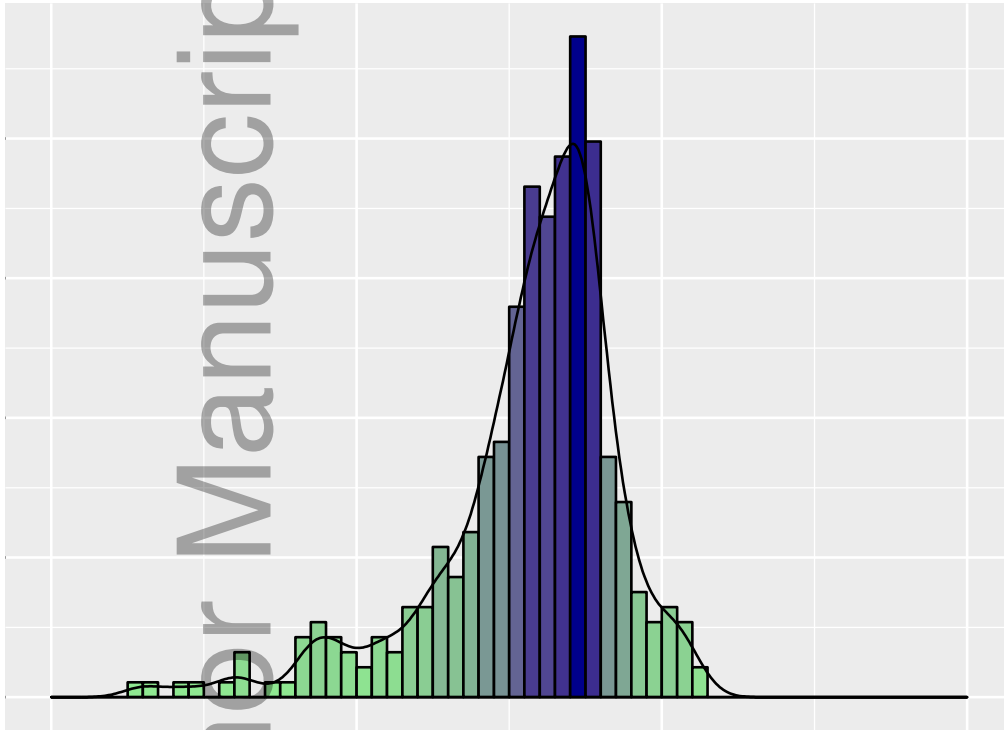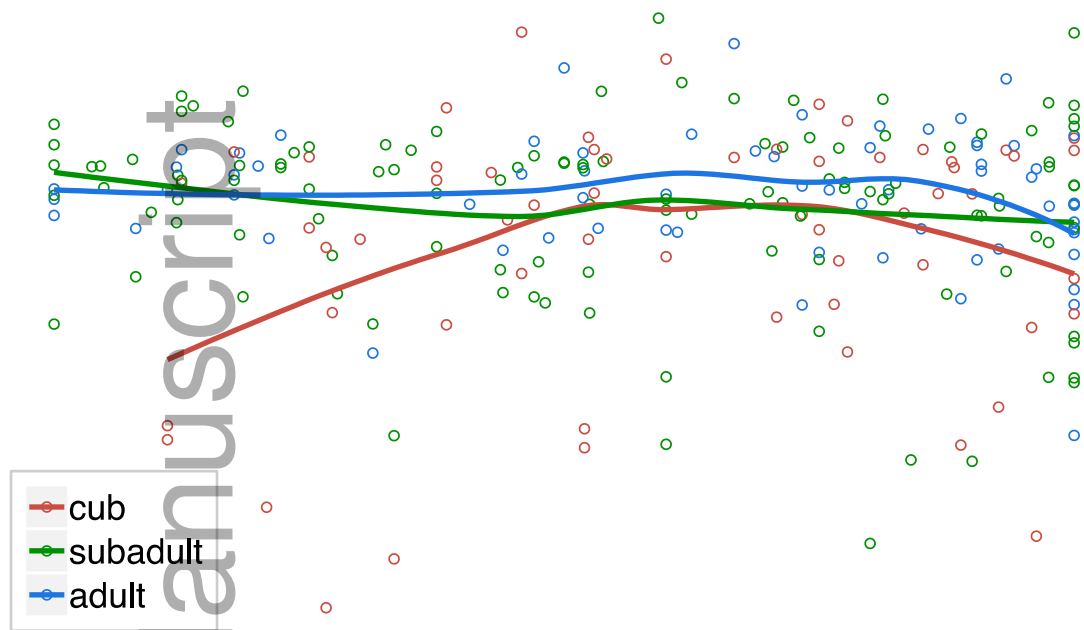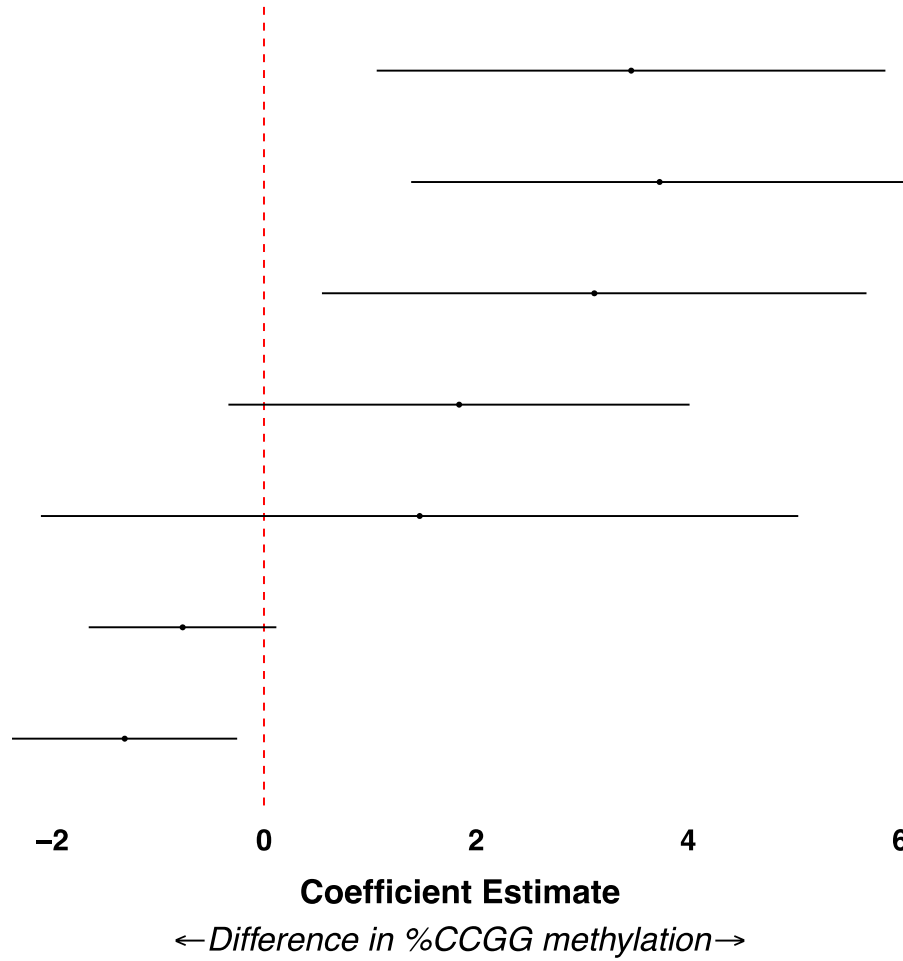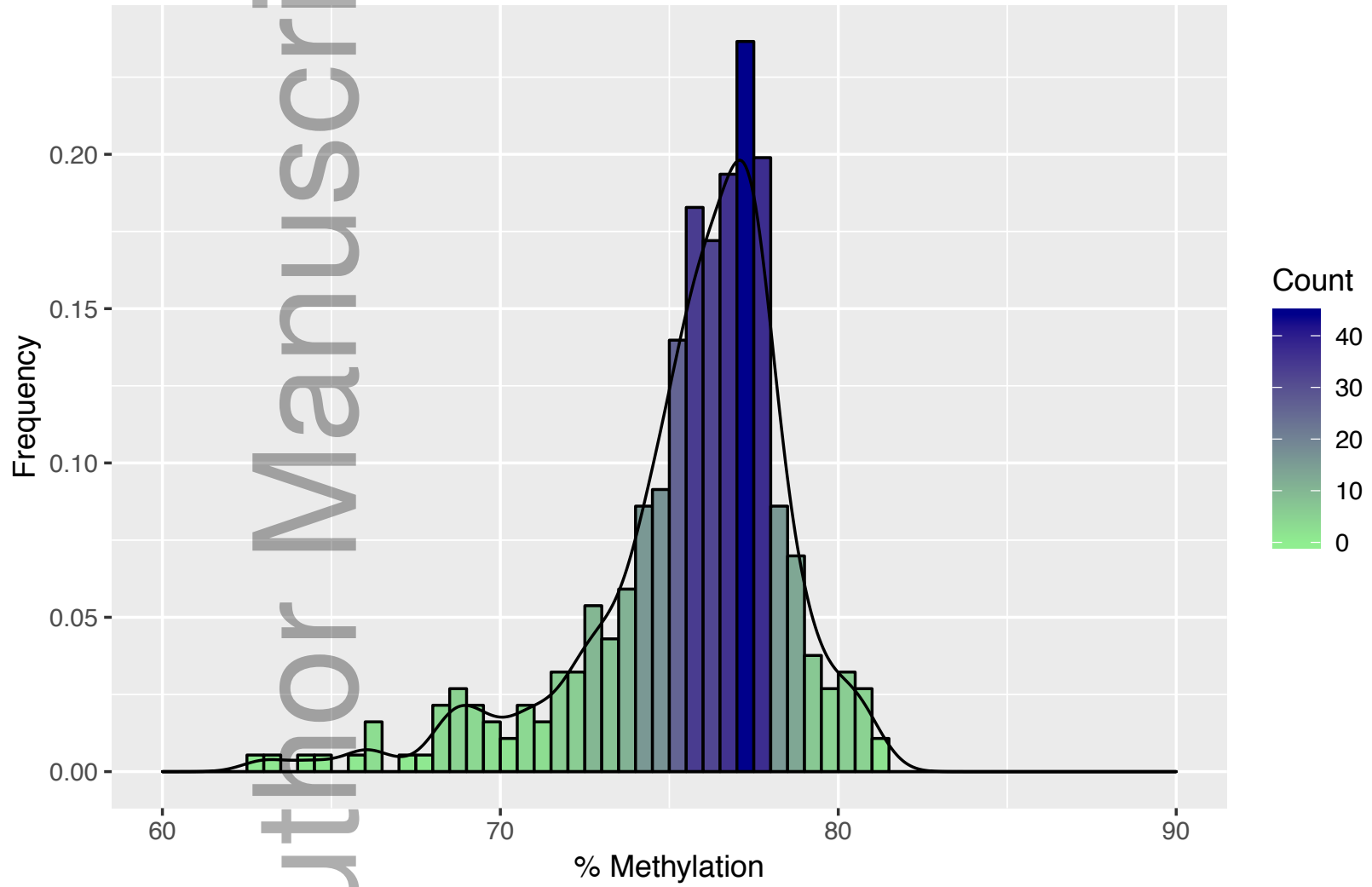
Figure 2 Hyena %CCGG methylation by standardized maternal rank (-1 lowest rank, and 1 highest rank) and stratified by age categories of cubs, subadults, and adults.
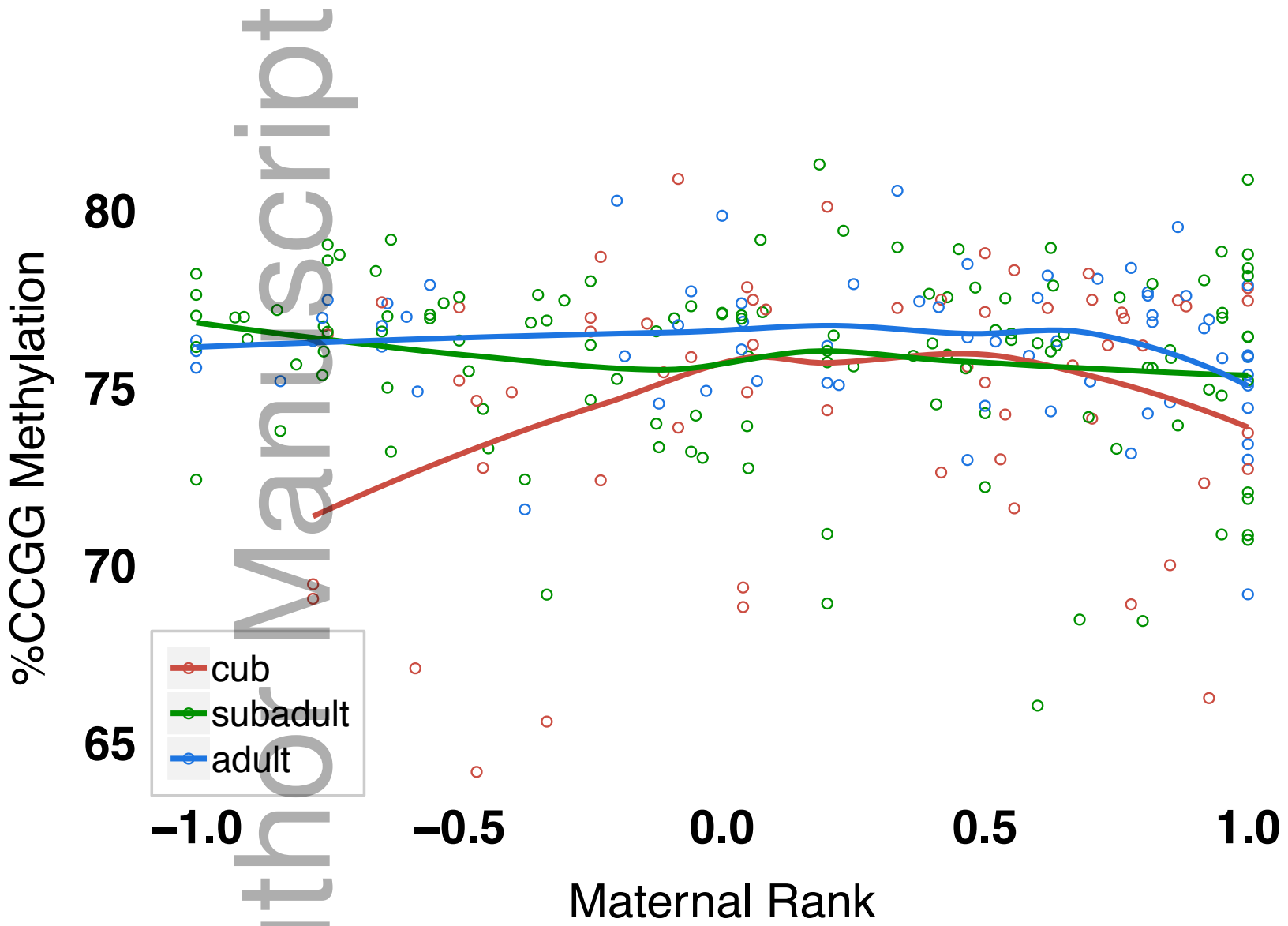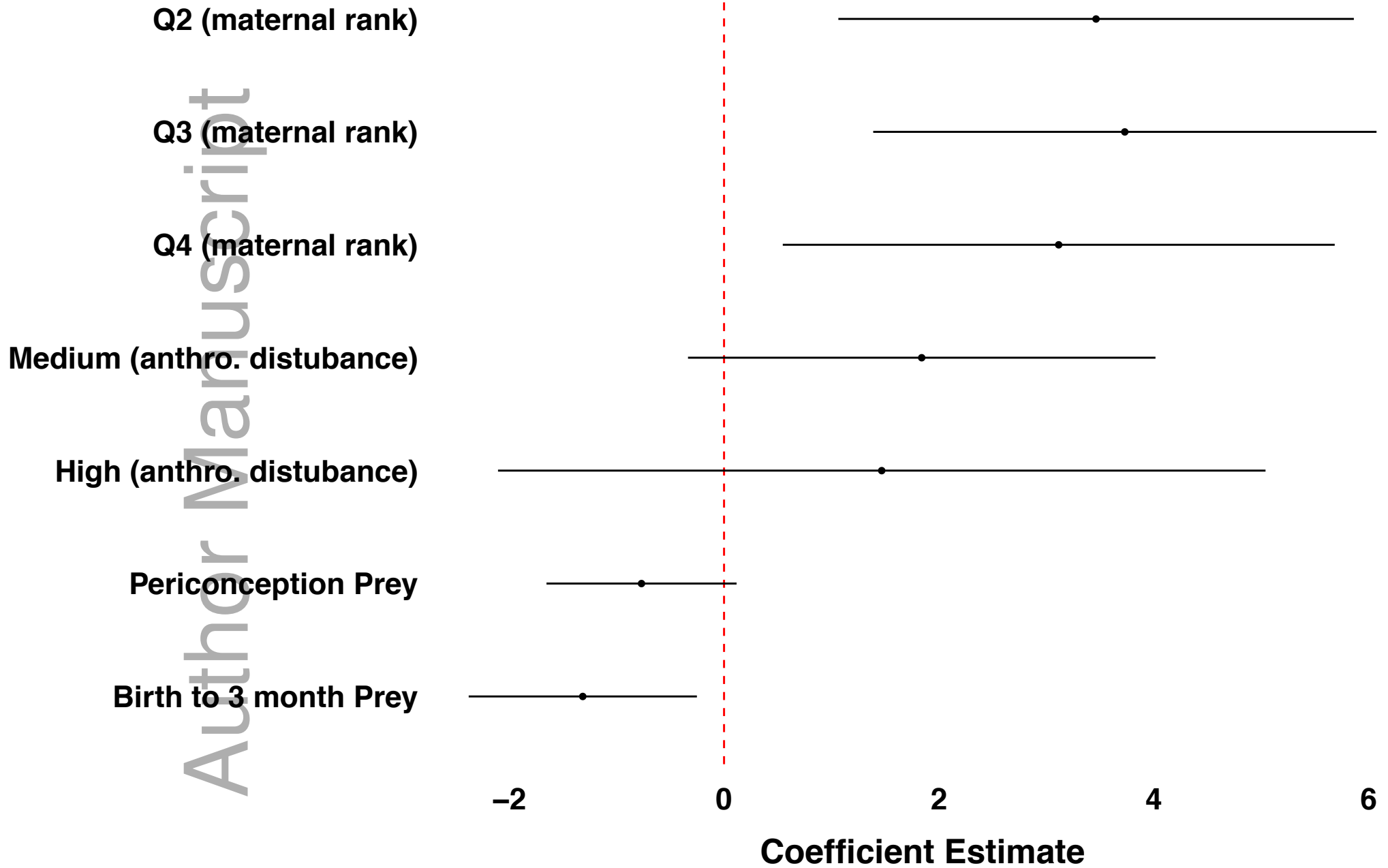
Figure 3 Beta estimates and 95% CI for %CCGG methylation from Model 2 in cubs. Model 2 included all significant explanatory variables from Model 1 including: maternal rank, anthropogenic disturbance, wild ungulate prey density at periconception and from birth to 3 months, as well offspring age at the time of darting and sex.

Coefficient Estimate

←Difference in %CCGG methylation→