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EPIGENETIC STUDIES IN ECOLOGY AND EVOLUTION

Resource base influences genome-wide DNA methylation levels in wild baboons (*Papio cynocephalus*)

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Abstract

Variation in resource availability commonly exerts strong effects on fitness-related traits in wild animals. However, we know little about the molecular mechanisms that mediate these effects, or about their persistence over time. To address these questions, we profiled genome-wide whole-blood DNA methylation levels in two sets of wild baboons: (i) 'wild-feeding' baboons that foraged naturally in a savanna environment and (ii) 'Lodge' baboons that had ready access to spatially concentrated human food scraps, resulting in high feeding efficiency and low daily travel distances. We identified 1014 sites (0.20% of sites tested) that were differentially methylated between wild-feeding and Lodge baboons, providing the first evidence that resource availability shapes the epigenome in a wild mammal. Differentially methylated sites tended to occur in contiguous stretches (i.e., in differentially methylated regions or DMRs), in promoters and enhancers, and near metabolism-related genes, supporting their functional importance in gene regulation. In agreement, reporter assay experiments confirmed that methylation at the largest identified DMR, located in the promoter of a key glycolysis-related gene, was sufficient to causally drive changes in gene expression. Intriguingly, all dispersing males carried a consistent epigenetic signature of their membership in a wild-feeding group, regardless of whether males dispersed into or out of this group as adults. Together, our findings support a role for DNA methylation in mediating ecological effects on phenotypic traits in the wild and emphasize the dynamic environmental sensitivity of DNA methylation levels across the life course.

Keywords: behavior/social evolution, ecological genetics, genomics/proteomics, mammals, primates

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Introduction

Despite a rich history of studies documenting the relationship between ecological and phenotypic variation in natural populations, we know surprisingly little about the molecular mechanisms that mediate these effects. Insight into these mechanisms is important for understanding how natural phenotypic variation emerges and how organisms cope with environmental change. Genomic approaches can contribute to these questions by identifying the genes and pathways involved in sensing and responding to selectively relevant ecological variation. For example, studies in eusocial insects have highlighted the impact of nutrient exposure on genome-wide gene regulation, as well as its contribution to the emergence of distinct castes within a hive or colony (Kucharski *et al.* 2008; Foret *et al.* 2012). At the same time, other studies have identified rapid gene expression responses to song in zebra finch (Drnevich *et al.* 2012; Whitney *et al.* 2014); a strong genome-wide signature of social status in hierarchical primates (Tung *et al.* 2012); and widespread transcriptional changes associated with mate choice in fish (Cummings *et al.* 2008). Together, such work points to a fundamental role for gene regulation in mediating physiological responses to

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environmental inputs. By altering the expression of genes in the genome, gene regulatory mechanisms permit a range of phenotypic values to arise from an otherwise static genome sequence.

Mounting evidence suggests that epigenetic marks, particularly DNA methylation (the best studied to date), play a significant role in mediating the gene regulatory response to environmental conditions. DNA methylation refers to the covalent addition of a methyl group to a cytosine base, and, in mammals, occurs most often at CG dinucleotides (known as 'CpG sites'). CpG sites are strongly enriched in regulatory sequences (e.g., gene promoters, gene bodies and CpG-dense regions known as 'CpG islands') where changes in methylation can impact the expression of nearby genes. For example, DNA methylation in promoter or enhancer regions can repress gene expression by interfering with transcription factor binding or by recruiting proteins that induce changes in chromatin accessibility (Klose & Bird 2006; Weber *et al.* 2007). Meanwhile, gene body methylation is often associated with increased gene expression and is thought to aid in transcriptional elongation (Jones 2012).

Genome-wide patterns of DNA methylation are first established during development and, once established, are faithfully transmitted across cell divisions throughout the life of the organism. However, environmental conditions can affect this process, either during development itself (when epigenetic patterns are known to be particularly sensitive: Tobi *et al.* 2009; Feil & Fraga 2011; Faulk & Dolinoy 2011) or later in life, when changes in DNA methylation help coordinate the cellular response to new environmental stimuli (Guo *et al.* 2011; Barrès *et al.* 2012; Pacis *et al.* 2015). Thus, changes in DNA methylation are thought to provide an avenue through which environmental inputs can stably alter gene expression levels and, as a consequence, mediate environmental effects on organism-level traits (Meaney & Szyf 2005; Jirtle & Skinner 2007; Feil & Fraga 2011).

The relationship between DNA methylation and diet (both caloric intake and dietary content) is particularly well-studied in this regard (Van den Veyver 2002; Heijmans *et al.* 2008; Carone *et al.* 2010; Moleres *et al.* 2013; Tobi *et al.* 2014). For example, in laboratory mice, maternal diet during pregnancy predicts offspring fur colour and susceptibility to diabetes – a relationship mediated by its stable effects on offspring methylation near the *agouti* gene, which in turn changes *agouti* gene expression (Klebig *et al.* 1995; Wolff *et al.* 1998). Long-lasting effects of diet and resource availability (which we refer to in combination as 'resource base') also affect DNA methylation patterns in humans. In Gambian populations that experience dramatic seasonal fluctuations in food availability, season of conception predicts offspring DNA methylation levels at several metastable epialleles (loci that show consistent, stable epigenetic patterns across tissues: Waterland *et al.* 2010; Dominguez-Salas *et al.* 2014). Similarly, individuals conceived during the Dutch Hunger Winter, a severe war-time famine in the Netherlands, exhibit stable differences in DNA methylation levels at individual growth-related genes (e.g., *IGF2*, *INSIGF*, and *IL10*) and on a genome-wide scale (Heijmans *et al.* 2008; Tobi *et al.* 2009, 2014). These studies also suggest that diet effects on DNA methylation can be acutely sensitive to timing: long-term changes in DNA methylation levels were only detectable in individuals exposed to the Dutch Hunger winter during the periconception period, but not later in pregnancy (Heijmans *et al.* 2008; Tobi *et al.* 2014; but see Tobi *et al.* 2009).

Variation in resource base, both during development and later in life, is also important in wild mammal populations, where it exerts potent effects on both fertility and mortality components of fitness (Altmann 1991; Gaillard *et al.* 2000; Beehner *et al.* 2006; Nussey *et al.* 2007; Hamel *et al.* 2009; Revitali *et al.* 2009). However, in contrast to human populations or laboratory model organisms, the role of DNA methylation in mediating these effects has not been investigated, leaving questions about the scope and timing of epigenetic sensitivity to the environment unanswered. To address this gap, we profiled genome-wide DNA methylation levels in a long-term study population of wild baboons in the Amboseli region of Kenya (Alberts & Altmann 2012). Specifically, we compared DNA methylation patterns in 'wild-feeding baboons' to those in 'Lodge group baboons'. Wild-feeding baboons walked 4–6 km per day, foraging in a dry savanna environment on widely distributed foods. In contrast, while Lodge group baboons resided in the same savanna ecosystem, they had access to spatially concentrated human food scraps. Lodge group baboons were therefore able to feed more efficiently and travel shorter distances each day to achieve the same caloric intake as wild-feeding animals (Muruthi *et al.* 1991; Bronikowski & Altmann 1996). In addition, they experienced reduced seasonal and annual variance in resource availability compared to wild-feeding animals. Previous work in Amboseli has documented striking behavioural and physiological differences between these groups. Lodge animals exhibited higher serum insulin, cholesterol and body fat levels compared to their wild-feeding counterparts (Muruthi *et al.* 1991; Altmann *et al.* 1993; Kemnitz *et al.* 2002). Further, Lodge juveniles grew faster and matured earlier than wild-feeding animals, suggesting that the combination of more stable resource availability, higher feeding efficiency and shorter travel distances translated

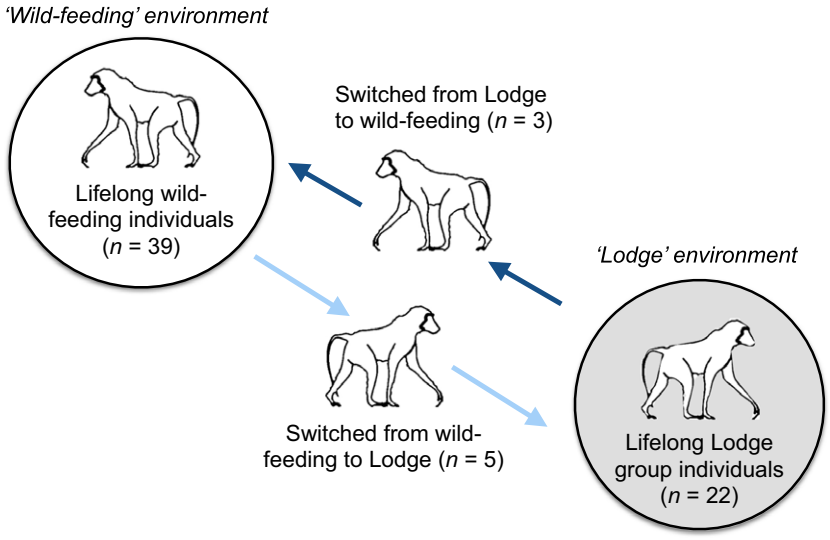

Fig. 1 Study design. The correspondence between resource base and all study subjects is depicted here. Our main differential methylation analyses focused on individuals that spent their entire life (from birth until the time of sampling) in a wild-feeding social group (n = 39: white circle) or in the Lodge group (n = 22: grey circle). Our analyses of the stability vs. plasticity of DNA methylation levels focused on individuals that switched resource base after natal dispersal (n = 8: switching baboons in the centre).

into measurable fitness advantages (Altmann & Alberts 2005).

Here, we investigated whether environmentally induced changes in DNA methylation levels might contribute to the known phenotypic differences between Lodge and wild-feeding animals. To do so, we investigated three sets of questions: (i) Do the differences in resource base-associated with the Lodge vs. wild-feeding conditions significantly predict DNA methylation levels? (ii) Are sites that are differentially methylated by resource base likely to be functionally important? and (iii) Is the signature of resource base stable or plastic over time, when environmental conditions change? For the third question, we drew on samples from eight male baboons that switched either from the Lodge to wild-feeding condition or from the wild-feeding to Lodge condition as a consequence of natal dispersal. We then asked whether predispersal (i.e., early life) or postdispersal (i.e., adult) resource base left a stronger signature on genome-wide DNA methylation patterns.

Materials and methods

Study subjects and sample collection

All study subjects were members of a long-term study population of yellow baboons (*Papio cynocephalus*) that has been monitored by the Amboseli Baboon Research Project (ABRP) for over four decades (Alberts & Altmann 2012). Our study focused on 69 animals from the ABRP population, including the following: (i) 39 baboons that resided in a wild-feeding group from birth until the time of sampling (29 males and 11 females); (ii) 22 baboons that either resided in Lodge group from birth until the time of sampling (11 males and 7 females) or gained access to the Lodge resource base early in their lives (4 females born prior to monitoring of Lodge group in 1982); (iii) Three males that were born in Lodge group and dispersed into a wild-feeding group following reproductive maturation and were sampled in a wild-feeding group as adults; and (iv) Five males that were presumed to have been born in a wild-feeding group and that dispersed into Lodge group following reproductive maturation and were sampled in Lodge (Fig. 1). For the three males that were born in Lodge group and sampled in a wild-feeding group, their early histories and dispersal events were directly observed. For the five males that were presumed born in wild-feeding groups but were sampled in Lodge group, we inferred their early histories based on two pieces of evidence. First, adult male baboons very rarely remain in their natal group to reproduce (Pusey & Packer 1987), and genetic analysis suggested that these adult males were unrelated to members of Lodge group other than their offspring (Altmann *et al.* 1996). Second, there were very few social groups associated with human food sources in the ecosystem at the time these males matured, suggesting wild-feeding origins for all or most of these animals. Further information about these eight postdispersal males, including the timing of dispersal relative to blood sampling, if known, is provided in Table S1 (Supporting information).

To investigate epigenetic differences between Lodge and wild-feeding animals, we combined genome-wide DNA methylation data from a previous study on statistical methods for differential DNA methylation analysis (n = 50 individuals; Lea *et al.* 2015b) with additional DNA methylation data generated to study resource base effects in this study (n = 19 individuals; see Fig. S7

(Supporting information) for quality control comparisons between the two data sets). All data were derived from whole-blood samples collected by the ABRP between 1989 and 2011, following well-established procedures (Altmann *et al.* 1996; Tung *et al.* 2009, 2011, 2015). Briefly, animals were immobilized by an anaesthetic-bearing dart delivered through a hand-held blow gun, and, following immobilization, were quickly transferred to a processing site for blood sample collection. Following sample collection, study subjects were allowed to regain consciousness in a covered holding cage until they were fully recovered from the effects of the anaesthetic. Upon recovery, study subjects were released near their social group and closely monitored. Blood samples were stored at the field site or at an ABRP-affiliated laboratory at the University of Nairobi until they were transported to the United States.

Generation and processing of genome-wide DNA methylation data

To measure genome-wide DNA methylation levels, we used a cost-effective, high-throughput sequencing approach known as reduced representation bisulphite sequencing (RRBS) (Meissner *et al.* 2008; Gu *et al.* 2011; Boyle *et al.* 2012). RRBS relies on two key steps: (i) digestion of genomic DNA with the enzyme *Msp1*, which produces DNA fragments that begin and/or end with an informative CpG site, and (ii) treatment of *Msp1*-digested DNA with the chemical sodium bisulphite, which leaves methylated cytosines intact but converts unmethylated cytosines to uracil (and ultimately thymine after PCR). Following high-throughput sequencing and mapping of all reads to a reference genome, site-specific DNA methylation levels can be estimated as the ratio of reads read as cytosine (reflecting an originally methylated version of the base) to the total number of mapped reads (reflecting both methylated and unmethylated versions of the base, i.e., reads read as either cytosine or thymine).

To construct RRBS libraries, we followed the protocol of Boyle and colleagues (Boyle *et al.* 2012). For each individual, we created a barcoded library from 180 ng of blood extracted baboon DNA, combined with 1 ng of unmethylated lambda phage DNA to assess the efficiency of the bisulphite conversion (see Supporting information). Each sample was sequenced to a mean depth (±SD) of 27.25 ± 13.62 million reads on the Illumina HiSeq 2000 platform. Following sequencing, we removed adapter contamination, low-quality bases and bases artificially introduced during library construction using the program Trim Galore! (Krueger 2015). We then used the program BSMAP (Xi & Li 2009) to map the trimmed reads to the olive baboon genome (PANU 2.0) and to extract the methylated read count and total read count for each individual and CpG site. Before performing differential methylation analyses, we filtered out constitutively hypermethylated and hypomethylated sites from our data set, as well as invariable sites and sites with low levels of mean coverage. Importantly, filtering for hypomethylation should reduce potential biases introduced from mapping yellow baboon RRBS reads to the olive baboon genome (see Supporting information and Fig. S1 for more details).

Testing for differences in DNA methylation levels at individual CpG sites

We first tested for a relationship between resource base and DNA methylation levels using data generated from individuals who had spent most or all of their lives (prior to sampling) in a wild-feeding group (n = 39) or the Lodge group (n = 22). To do so, we used the binomial mixed effects approach implemented in the program MACAU (Lea *et al.* 2015b). This approach allowed us to control for kinship in our data set and to work directly with the raw count data – two features that maximize power in bisulphite sequencing data sets. Specifically, for each CpG site, we used MACAU to model DNA methylation levels as a function of the fixed effects of resource base (Lodge or wild-feeding), sex, age of the animal, number of years since blood sample collection, and bisulphite conversion rate (see Table S2, Supporting information for all covariate values). We also included a random effect that accounts for genetic relatedness among individuals (see Supporting information and Lea *et al.* 2015b). For each CpG site tested, we extracted the *P*-value associated with the resource base term and corrected for multiple hypothesis testing using the false discovery rate (FDR) approach implemented in the R package *qvalue* (Storey & Tibshirani 2003; Dabney & Storey 2015). We considered a CpG site to be differentially methylated by resource base (referred to below as 'resource base-associated') if it passed a 10% FDR threshold.

Because DNA methylation patterns are highly cell type specific (Reinius *et al.* 2012; Roadmap Epigenomics Consortium *et al.* 2015), we also investigated whether differences in whole-blood cell-type composition between Lodge and wild-feeding animals could confound our analysis. To do so, we drew on two data sets: (i) cell-type proportion data generated from manual counts of Giemsa-stained blood smears (for 15 Lodge and 20 wild-feeding animals) and (ii) genome-wide, cell type-specific DNA methylation data from a previous study of human whole blood (Reinius *et al.* 2012; Jaffe 2015). We used these data to first test whether resource base predicted cell-type composition and then to

investigate whether resource base-associated sites were more likely to exhibit cell type-specific DNA methylation patterns, which would suggest a potential confound (Supporting information).

Enrichment of differentially methylated sites by genomic annotation

We hypothesized that, if shifts in DNA methylation are part of a coordinated regulatory response to resource base, these epigenetic changes should be biased towards regions of the genome that control gene expression, and should be targeted towards genes involved in similar biological processes. To test these hypotheses, we evaluated whether our data were consistent with four predictions. Specifically, we expected resource base-associated sites to be:

1 over-represented in putatively functional gene regulatory elements (i.e., gene bodies, promoters, CpG islands, CpG island shores or enhancers) and under-represented in regions of the genome with no known regulatory function;
2 over-represented in chromatin states associated with active gene transcription and under-represented in chromatin states associated with gene repression;
3 more likely to fall in or near genes expressed in whole blood, compared to genes not expressed in blood; and
4 enriched near genes involved in coherent biological pathways and processes.

To test prediction (1), we used publicly available annotation tracks for the olive baboon genome to assign each resource base-associated CpG site to one of the following categories: gene body, promoter, CpG island, CpG island shore, H3K4me1-marked enhancer or unannotated (see Supporting information and Fig. S2 for further information about category definitions). For each category, we used Fisher's exact test to test for significant over- or under-enrichment of resource base-associated sites relative to chance expectations. Importantly, we defined chance expectations based on the CpG sites that we actually profiled in our data set, which are themselves enriched for putatively functional regions of the genome (Fig. S7, Supporting information). Thus, significant over- or under-enrichment of resource base-associated sites would indicate that resource base-associated sites are *even more* likely to fall in a given genomic compartment than other sites captured by the RRBS protocol.

To test prediction (2), we drew on chromatin state annotation data generated by the NIH Roadmap Epigenomics Project (Roadmap Epigenomics Consortium *et al.* 2015) for human peripheral blood mononuclear cells. Chromatin states are defined by combinations of histone marks (acetylation or methylation) and provide information about the transcriptional activity and regulatory element function of the associated DNA. For example, actively expressed gene bodies are associated with a chromatin state defined by H3K36me3 marks, whereas repressed genes are associated with a chromatin state defined by H3K27me3 marks. Importantly, histone marks tend to be highly conserved between humans and closely related primates, such as chimpanzees and rhesus macaques (Zhou *et al.* 2014); the baboon lineage diverged from the human lineage at the same time as rhesus macaques, supporting the overall accuracy of using Roadmap Epigenomics chromatin states here. We therefore assigned each resource base-associated CpG site to one of 15 chromatin states (Fig. S3, Supporting information). We then investigated the degree to which resource base-associated sites were over- or under-enriched in each chromatin state. As above, we again used Fisher's exact tests against the background of the CpG sites included in our RRBS data set (not compared to the whole genome).

To test predictions (3) and (4), which rely on gene-level information, we first assigned each CpG site to a particular gene if it occurred in the gene body or within 10 kb of the gene transcription start site (TSS) or end site (TES; see also Fig. S4, Supporting information for results based on alternative criteria for CpG assignment to genes). To test prediction (3), we then categorized all genes as either not expressed in whole blood or blood-expressed (based on whether they were included in a whole-blood RNA-seq data set also from the Amboseli baboons, Tung *et al.* 2015). We used Fisher's exact test to ask whether CpG sites assigned to blood-expressed genes were more likely to be differentially methylated by resource base, compared to CpG sites assigned to unexpressed genes.

Finally, to test prediction (4), we performed categorical enrichment analysis using publicly available gene annotations (Kyoto Encyclopedia of Genes and Genomes (KEGG): Ogata *et al.* 1999) and the GeneTrail analysis software (Backes *et al.* 2007). Here, we focused only on genes associated with differentially methylated sites (as defined above) and tested for over-representation of genes that fall within specific pathways, compared to chance expectations. To do so, we used hypergeometric tests followed by FDR correction (Benjamini & Hochberg 1995).

Testing for differences in DNA methylation levels at metabolic pathways

Given the known differences in diet, activity patterns, and physiology between Lodge and wild-feeding

animals, we were particularly interested in whether metabolism-related genes showed an epigenetic signature of resource base. To specifically address this question, we focused on CpG sites near (in the gene body or within 10 kb of the gene TSS or TES) genes involved in 36 KEGG pathways related to the metabolism of food or to energy balance (Fig. S5, Supporting information) (Ogata *et al.* 1999). These pathways were chosen *a priori* because of their relevance to the phenotypic differences between wild-feeding and Lodge animals. We used the R package 'GlobalTest' (Goeman *et al.* 2004) to ask whether linearly transformed methylation levels (Supporting information) from CpG sites near genes involved in metabolism-related pathways displayed a signature of resource base. This approach asks whether samples with similar DNA methylation patterns (at predefined sets of sites) also have similar resource base labels (i.e., Lodge or wild-feeding), using a framework similar to penalized logistic regression. Thus, the level of analysis is shifted from individual CpG sites to sets of CpG sites associated with putatively similar functions, allowing us to specifically test pathway-based predictions (Goeman *et al.* 2004). We corrected all GlobalTest *P*-values for multiple hypothesis testing using the R package *qvalue* (Storey & Tibshirani 2003; Dabney & Storey 2015).

Identification of differentially methylated regions (DMRs)

Spatially contiguous stretches of differentially methylated sites (often termed 'differentially methylated regions', or DMRs) are more likely to have functional effects on gene expression than differentially methylated sites that occur in isolation (Lister *et al.* 2009; Hansen *et al.* 2011; Jaffe *et al.* 2012). To identify DMRs in our data set, we focused on resource base-associated sites (detected at a 10% FDR) that had at least one other measured CpG site within a 2-kb window centred on the focal site (following the precedent for window size used in Lister *et al.* 2009 and Hansen *et al.* 2012). For sites that met this criterion, we counted the absolute number of nearby sites that also exhibited evidence for differential methylation, at a less conservative 20% FDR threshold. We defined DMRs as a cluster of at least 3 resource base-associated sites. We chose this cut-off because clusters of this size were unlikely to occur by chance in permuted data (Supporting information and Fig. S6). Specifically, despite relaxing the FDR threshold for identifying CpG sites close to the original resource base-associated sites, our criteria for identifying DMRs results in a relatively stringent FDR threshold of 6.5% FDR. Finally, we collapsed any DMRs with overlapping boundaries into a single, longer DMR.

Testing the effects of PFKP promoter methylation on gene expression levels

Our analyses revealed one particularly large DMR at the promoter region of the phosphofructokinase gene (*PFKP*). This DMR stretched across 192 CpG sites, including 30 sites associated with resource base at a 10% FDR (Fig. 4A). Because *PFKP* is involved in the rate-limiting step of glycolysis and has been previously implicated in obesity-related traits (Ehrich *et al.* 2005; Scuteri *et al.* 2007), we were interested in understanding whether *PFKP* promoter methylation alone was sufficient to drive differences in gene expression. This relationship is implicitly assumed by arguments linking environmental variation to phenotypic variation via epigenetic mechanisms, but is rarely tested in practice.

To test this hypothesis, we used an experimental reporter gene assay in which we cloned 817 bp of the *PFKP* promoter (containing 72 CpG sites) into a CpG-free vector backbone that contains the luciferase reporter gene (pCpGL, Klug & Rehli 2006). After growing up the *PFKP*-pCpGL construct in competent *E. coli* GT115 cells (InvivoGen), we subjected the purified plasmid to one of three treatments: (i) methylation of all 72 CpGs in the *PFKP* promoter region via treatment with *M.SssI* (a methyltransferase that targets all CG sequence motifs, resulting in a completely methylated *PFKP* promoter); (ii) methylation of 13 CpGs in the *PFKP* promoter via treatment with *HhaI* (a methyltransferase that targets only CGCG sequence motifs, resulting in a partially methylated *PFKP* promoter); and (iii) a mock treatment (water substituted for the methyltransferase enzyme, resulting in a completely unmethylated *PFKP* promoter).

We transfected four replicates of each treatment condition into the human K562 myeloid cell line and incubated the transfected cells for 24 h ($n = 12$ total transfection experiments). To control for transfection efficiency, a vector containing Renilla luciferase was transfected in parallel. Postincubation, cells were assayed for luciferase activity using a dual-luciferase reporter assay kit (Promega), and luciferase expression was normalized using measures of cotransfected Renilla activity. Finally, we tested for an effect of DNA methylation at the *PFKP* promoter on luciferase gene expression using pairwise Wilcoxon rank-sum tests. Additional details on our experimental procedures are provided in the Supporting information.

Investigating the stability or plasticity of DNA methylation levels for individuals that switched between resource bases

Finally, we tested two alternative hypotheses about DNA methylation patterns in the 8 males that switched

resource base as a consequence of natal dispersal. First, we hypothesized that if resource base exerts stable, long-term effects on DNA methylation patterns, switching individuals should resemble their natal group members (Lodge or wild-feeding) rather than the group to which they belonged at the time of sampling (Fig. 5A). Alternatively, if resource base-associated DNA methylation patterns are largely plastic in response to prevailing conditions, we hypothesized that switching individuals should exhibit DNA methylation patterns that reflect their resource base at the time of sampling rather than their natal group (Fig. 5B).

To differentiate between these two possibilities, we built a support vector machine (SVM) classifier, a machine-learning approach used for class prediction from high dimensional data (Cortes & Vapnik 1995). This classifier used DNA methylation data to distinguish between individuals that spent all (or the vast majority) of their lives either in a wild-feeding group or in Lodge group (n = 61 individuals). As predictive features for this model, we included the 334 840 CpG sites that were not associated with age, sex, bisulphite conversion rate or sample age at a nominal P-value of 0.05. We chose this global approach (rather than using significantly differentially methylated sites only) because it allowed us to include sites that may be truly affected by resource base, but did not pass the genome-wide significance threshold in the site-by-site analysis. Additionally, using all sites ensured that the model classification accuracy was not biased by using features that had already been associated with the response variable in a previous analysis of the same data set (doing so can result in erroneously high classification accuracy even from completely random data: Hastie *et al.* 2009). Because SVMs cannot work on binomially distributed count data, we linearly transformed our data before building the SVM (Supporting information).

Finally, we used the resulting SVM to ask whether individuals that switched resource base more closely resembled their preswitch or postswitch conspecifics. To do so, we used the fitted model to predict the resource base of the 8 individuals that dispersed between groups (using DNA methylation data from these 8 individuals, for the same 334 840 CpG sites).

Ethics statement

The data used in this study were generated from wild baboon samples, collected in the Amboseli region of Kenya. This research was conducted under the authority of the Kenya Wildlife Service (KWS), the Kenyan governmental body that oversees wildlife (current permit numbers NCST/RCD/12B/012/57 to Jenny Tung, NCST/5/002/R/777 to Susan Alberts, and NCST/5/002/R/776 to Jeanne Altmann). As the animals are members of a wild population, KWS requires that we do not interfere with injuries to study subjects inflicted by predators, conspecifics or through other naturally occurring events. Permission to perform temporary immobilizations (for blood sample collection) was granted by KWS; further, these immobilizations were supervised by a KWS-approved Kenyan veterinarian, who monitored anaesthetized animals for hypothermia, hyperthermia and trauma (no such events occurred during our sample collection efforts). Observational and blood sample collection protocols were approved though IACUC committees at Duke University (current protocol is A020-15-01 to Jenny Tung and Susan C. Alberts).

Results

Genome-wide DNA methylation levels contain a signature of resource base

We found that DNA methylation patterns in our full data set (i.e., after quality control, but before filtering for constitutively hypermethylated, hypomethylated or invariant sites) recapitulated typical patterns observed in mammalian genomes. Specifically, most of the genome was hypermethylated, with the exception of H3K4me1-marked enhancers, promoters and CpG islands (Fig. S7, Supporting information). Further, DNA methylation levels near the transcription start sites (TSS) of expressed genes were inversely related to their expression levels (Fig. S7, Supporting information).

After filtering, we investigated DNA methylation levels at over half a million CpG sites in the baboon genome (n = 535 996 sites). As expected when using RRBS (Gu *et al.* 2011; Boyle *et al.* 2012), many of these sites occurred in CpG-rich regions, particularly gene bodies (224 553 sites), promoters (25 730 sites), CpG islands (57 461 sites) and CpG island shores (117 226 sites; Fig. S7, Supporting information). Further, this data set encompasses many putatively functional regions of the genome, as at least one CpG was measured in 66% of genes, 28% of promoters, 40% of CpG islands, 44% of CpG island shores and 11% of enhancers. Within our filtered set, we identified 1014 sites (at a 10% FDR) that were differentially methylated between lifelong wild-feeding and Lodge group animals (Fig. 2; see also Fig. S8, Supporting information for a power analysis of our data set). We did not detect significant effects of sex on DNA methylation levels, or significant sex by resource base interaction effects, consistent with previous studies that have identified weak or no sex effects in human blood (Eckhardt *et al.* 2006; Lam *et al.* 2012). Also in line with previous studies (Tobi *et al.* 2014), we

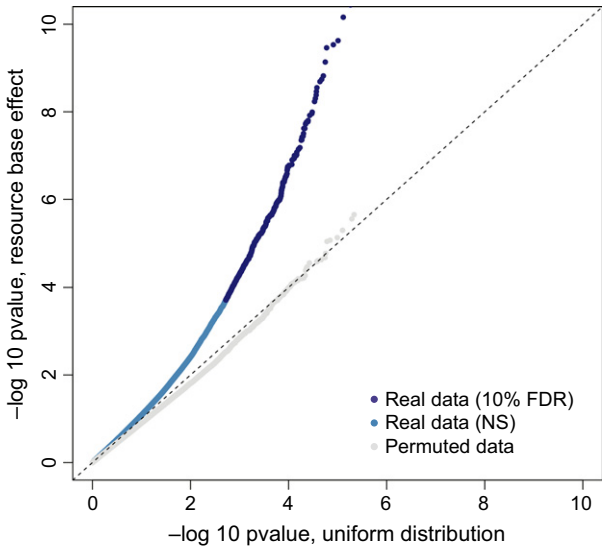

Fig. 2 Resource base influenced genome-wide DNA methylation levels. QQ-plot comparing the cumulative distribution of *P*-values from a uniform distribution against the cumulative distribution of (i) *P*-values generated from our main model, which tests for effects of resource base on site-specific DNA methylation levels (plotted in light and dark blue); and (ii) *P*-values generated from the same model when resource base values (Lodge or wild-feeding) were permuted. If resource base did not affect DNA methylation levels, we would expect the *P*-values associated with our main model (blue points) to follow a uniform distribution and to fall along the x = y line. The observed deviation from the x = y line suggests a pervasive effect of resource base on DNA methylation levels in the real data. Note that deviation from the x = y line at higher *P*-values (beginning around $P = 0.1$) suggests that more sites are affected by resource base than we could detect in our sample size of 61 individuals, but do not reach genome-wide significance (thus, our nonsignificant set of sites is a mix of true positives and true negatives). In contrast, when resource base values were permuted across individuals, the resulting *P*-values were roughly uniformly distributed, as expected, and fell along the x = y line.

did not observe strong directional bias for the 1014 differentially methylated sites (Fig. S9, Supporting information). Importantly, our analyses of cell-type composition and cell type-specific DNA methylation data indicate that our results are unlikely to be explained by cell-type heterogeneity effects (Supporting information and Fig. S10).

Sites associated with resource base are enriched in functionally important regions of the genome

CpG sites associated with differences in resource base were nonrandomly distributed in the genome. Specifically, they were enriched in putative enhancers ($P = 9.70 \times 10^{-3}$) and gene promoters ($P = 3.66 \times 10^{-3}$) and underrepresented in functionally unannotated regions of the genome ($P = 8.96 \times 10^{-10}$; Fig. 3). Further, they were more likely to occur near genes expressed in whole blood than near unexpressed genes (odds ratio = 1.51, $P = 5.49 \times 10^{-7}$; see also Supporting information and Fig. S11). More fine-grained analyses of chromatin states also indicated a role for differentially methylated sites in the active regulation of genes: differentially methylated sites were more likely to occur in chromatin states associated with active gene transcription in blood cells, including chromatin states designated as 'active TSS' ($P = 2.78 \times 10^{-4}$), 'flanking active TSS' ($P = 1.44 \times 10^{-2}$), 'strong transcription' ($P = 5.24 \times 10^{-3}$) and 'enhancer' ($P = 1.28 \times 10^{-3}$). In contrast, differentially methylated sites were strongly under-enriched in a chromatin state indicative of gene repression ('repressed polycomb', $P = 5.49 \times 10^{-3}$).

Resource base-associated CpG sites are enriched in specific biological pathways

We observed two pieces of evidence that resource base-associated CpG sites were concentrated in specific biological pathways. First, while no KEGG pathways were enriched at a 10% FDR threshold, a more relaxed 20% FDR threshold revealed that resource base-associated sites were enriched near genes involved in 5 KEGG pathways: the T-cell receptor and B-cell receptor signalling pathways, axon guidance, phosphatidylinositol signalling and insulin signalling. Second, our GlobalTest analyses revealed patterns of differential methylation associated with carbohydrate metabolism (galactose: $P = 1.29 \times 10^{-5}$; fructose and mannose: $P = 1.79 \times 10^{-4}$; and the glycolysis pathway: $P = 2.94 \times 10^{-4}$); amino acid metabolism (glycine, serine and threonine: $P = 4.21 \times 10^{-3}$; tryptophan: $P = 0.018$); insulin signalling ($P = 0.012$); and the breakdown of other dietary components (propanoate, $P = 0.018$; all GlobalTest results reported a 10% FDR threshold; see Fig. S5, Supporting information). For pathways that included the *PFKP* gene (specifically, the three carbohydrate metabolism pathways listed above), the observed effect of resource base on DNA methylation levels appears to have been driven almost entirely by differential methylation at *PFKP* (Fig. S5, Supporting information).

DMRs occur more often than expected by chance, and near a key metabolic gene

We identified 87 2-kb windows that met our criteria for differentially methylated regions, compared to only 6 such windows observed on average in permuted data (equivalent to a 6.5% FDR; see Fig. S6, Supporting information). These 87 windows collapsed into 29

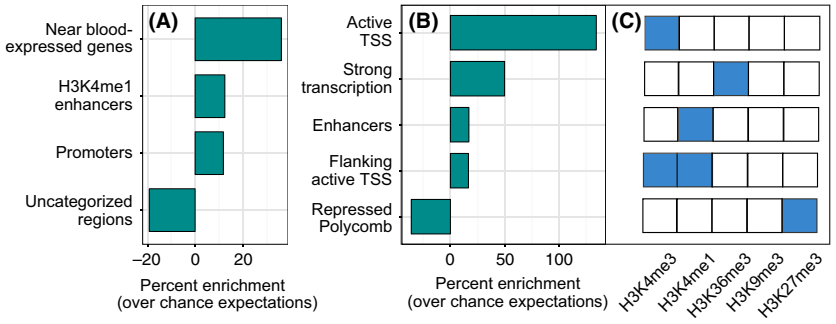

Fig. 3 Sites affected by resource base were enriched in functionally important regions of the genome. (A) Sites associated with resource base were more likely to occur within 10 kb of genes expressed in whole blood, in regions homologous to H3K4me1-marked enhancers in humans, and in gene promoters. They were significantly under-represented in regions of the genome with no known functional role. (B) Sites associated with resource base were also nonrandomly distributed across chromatin states. 'Active TSS' and 'Flanking Active TSS' reflect the transcription start sites of actively expressed genes; 'Strong transcription' reflects gene bodies of highly expressed genes; 'Enhancers' reflects regulatory elements that interact with promoters of expressed genes; and 'Repressed Polycomb' reflects the promoter and gene bodies of silenced/unexpressed genes. In both (A) and (B), only regions/states with significant over/under-enrichment are shown (see Fig. S3, Supporting information for the full set of regions/states) (C) Histone marks associated with each of the chromatin states presented in panel B (based on Roadmap Epigenomics data (Roadmap Epigenomics Consortium *et al.* 2015)). Each chromatin state is defined by the presence (blue square) or absence (white square) of the histone modifications shown below.

distinct, longer DMRs, the largest of which fell within the promoter region of an insulin-sensitive gene that encodes the rate-limiting enzyme in glycolysis (Lo *et al.* 2013; Webb *et al.* 2015). For 90% of the 192 sites, we tested in this region, *PFKP* was more highly methylated in wild-feeding individuals than in Lodge group baboons (Fig. 4A; no site was significantly more highly methylated in Lodge group animals), suggesting that *PFKP* expression may be downregulated in less resource-rich environments.

In support of this possibility, our reporter assay experiments confirmed that complete methylation of all CpGs in the *PFKP* promoter region (n = 72 CpGs in the region we tested; Supporting information) suppressed luciferase expression levels relative to fully unmethylated *PFKP* promoter constructs (Wilcoxon rank-sum test, W = 16, P = 0.014). Furthermore, even methylation of a minority of CpGs in the *PFKP* promoter (n = 13 CpGs) produced a graded reduction in gene expression levels, intermediate between the fully methylated and fully unmethylated versions (Wilcoxon rank-sum test, W = 14, P = 0.057 for comparison between fully methylated and partially methylated constructs; Fig. 4B).

Individuals that switched between resource bases more closely resembled wild-feeding individuals, regardless of the direction of the switch

Finally, we built a support vector machine (SVM) classifier (trained on 334 840 CpG sites) that discriminated between Lodge and wild-feeding individuals with 82% accuracy. We used this model to classify switching individuals based on their DNA methylation levels to test whether switching individuals would be (i) grouped with their natal conspecifics, suggesting that early life (i.e., predispersal) resource base drives variation in DNA methylation levels; or (ii) grouped with their conspecifics at the time of sampling, suggesting that prevailing conditions are more important than past history (Fig. 5).

We did not find evidence in support of either of these hypotheses. Instead, we found that the DNA methylation patterns of most switching males (7 of 8) were consistently predicted to be more like wild-feeding animals. This pattern held regardless of whether we considered males that immigrated from a wild-feeding group to Lodge group, or males that immigrated from Lodge group to wild-feeding groups. Only one male – an individual who moved from a wild-feeding group into Lodge group – violated this pattern, primarily because our model could not consistently classify him with either wild-feeding or Lodge feeding individuals (Fig. 5).

Discussion

Ecological variation, experienced throughout life, can have lasting and dramatic effects on trait variation. However, the molecular mechanisms that mediate these effects remain largely unexplored, especially in natural populations. Here, we present the first evidence that resource base – an environmental variable with known effects on activity patterns, growth rates, insulin levels, and body fat percentages in our study population – influences DNA methylation levels in a wild mammal

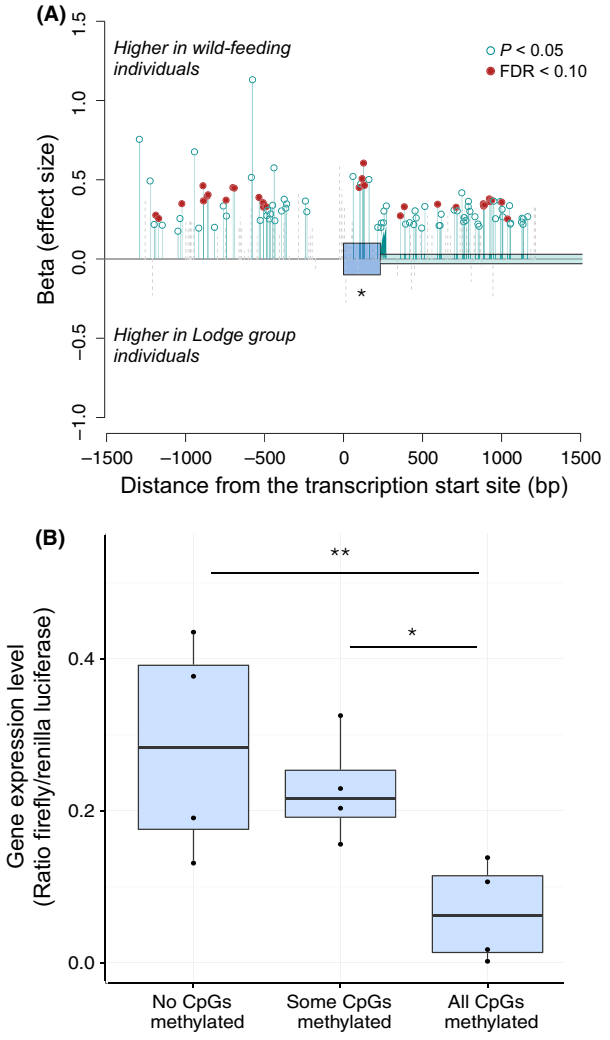

Fig. 4 Wild-feeding baboons exhibited consistently higher levels of DNA methylation at the phosphofructokinase (*PFKP*) promoter, where methylation suppresses gene expression in reporter gene assays. (A) The magnitude and direction of the effect of resource base on DNA methylation levels are plotted for all sites tested within 1.5 kb of the *PFKP* transcription start site. Sites with evidence for an effect of resource base at a nominal *P*-value of 0.05 are shown as green lines with open green dots, and at a 10% FDR as green lines with filled red dots. All other sites are shown as grey lines. The first exon is denoted by a blue box, and the translation start site is denoted with a black asterisk. (B) Firefly luciferase expression levels (normalized by renilla luciferase expression levels) are plotted for 4 replicates per condition. Results from Wilcoxon signed rank tests are shown as follows: ** $P = 0.014$ for comparison between no CpGs methylated and all CpGs methylated; * $P = 0.057$ comparison between no CpGs methylated and some CpGs methylated.

(Muruthi *et al.* 1991; Altmann *et al.* 1993; Kemnitz *et al.* 2002; Altmann & Alberts 2005). Specifically, we identified over a thousand differentially methylated CpG sites, as well as 29 differentially methylated regions, that differed between wild-feeding and Lodge group baboons. Our results support the importance of DNA methylation in translating signals from the environment into changes in gene regulation within cells.

The functional relevance of differential methylation at resource base-associated sites

Several pieces of evidence suggest that the changes in DNA methylation we observed are targeted, coordinated and likely to exert downstream effects on gene regulation. Specifically, differentially methylated sites were more likely to occur in promoter and enhancer regions; near genes expressed in blood, the tissue we sampled; and at stretches of DNA marked by transcriptionally active chromatin states. Further, differential methylation consistently occurred near genes involved in metabolism and insulin signalling, one of the known differences between Lodge and wild-feeding baboons in Amboseli (Kemnitz *et al.* 2002).

Because current methods for functional validation (e.g., reporter assay experiments) are not feasible on a genome-wide scale, we focused on validating the functional role of changes in DNA methylation at the largest identified DMR (in the promoter region of *PFKP*). However, several of the additional DMRs we identified fall near genes with relevance to metabolism and energy balance and may also contribute to organism-level differences (Table S3, Supporting information). For example, we identified a DMR in *KCNIP4*, where genetic variants have been previously associated with obesity-related traits (Comuzzie *et al.* 2012). In addition, we identified a DMR in the 5′ UTR of *TPM1*, where genetic variation has been associated with platelet count and volume (Soranzo *et al.* 2009; Gieger *et al.* 2011), both of which are biomarkers of obesity and metabolic syndrome (Coban *et al.* 2005; Jesri *et al.* 2005; Tavil *et al.* 2007). Together, these results point towards a model in which easy access to resources alters metabolic processes in Lodge group animals, at least in part through targeted changes in DNA methylation. Further work is needed to assess the causal effects of changes in DNA methylation on gene expression at these loci, and more generally to improve methods for high-throughput, genome-wide functional validation.

By far, the most striking DMR we identified fell near a gene coding for an isoform of phosphofructokinase, which catalyses the irreversible, committed step of glycolysis. Our reporter assay experiments indicate that lower levels of *PFKP* promoter methylation are sufficient to drive higher levels of *PFKP* expression (Fig. 4B). In combination with work in laboratory mice, these results suggest a possible avenue through which regulatory changes at *PFKP* may contribute to organism-level

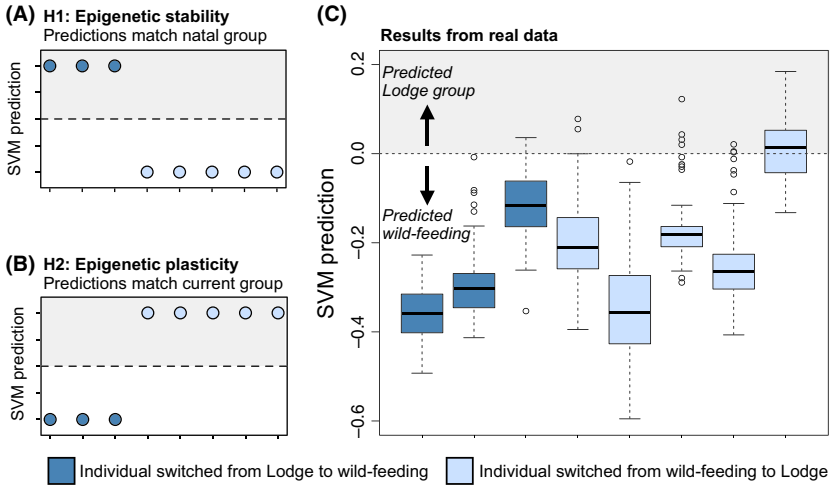

Fig. 5 Individuals that switched resource base more closely resembled lifelong wild-feeding individuals, regardless of the direction of the switch. (A–B) Predicted and (C) observed results when an SVM classifier that distinguished between wild-feeding and Lodge individuals was applied to DNA methylation data from individuals that switched resource base at natal dispersal (for all plots, grey background = predicted Lodge group, white background = predicted wild-feeding). We hypothesized that DNA methylation patterns in switching individuals would consistently (A) resemble their early life group mates or (B) resemble their current group mates. However, we observed (C) that regardless of the resource base history of switching individuals, they consistently resembled lifelong wild-feeding individuals (represented as an SVM prediction value below 0). Results in C are shown as boxplots (distributions of predicted values for each individual) because we randomly subsampled our data (50 separate subsamples) to create balanced training sets before predicting the resource base of switching individuals.

changes – namely, body fat mass accumulation – in Lodge animals. Specifically, several studies have demonstrated that mice that become obese on high carbohydrate diets exhibit increased levels of the phosphofructokinase enzyme relative to mice that did not become obese, but ingested similar numbers of calories (Yamini *et al.* 1992; Dourmashkin *et al.* 2005). Further, mice with experimentally reduced expression levels of the *PFKM* gene (the muscle isoform of phosphofructokinase) exhibit greatly reduced levels of both lipogenesis (the process by which energy is stored as fat) and overall body fat (Getty-Kaushik *et al.* 2010). Together, these studies argue that phosphofructokinase activity is stimulated by a high carbohydrate diet and consequently favours increased fat accumulation. Previous work in Amboseli has only tested for differences in protein content and overall energy intake between Lodge and wild-feeding animals (revealing that protein intake is higher in wild-feeding animals, while energy intake does not differ: Muruthi *et al.* 1991). However, Lodge animals were likely to have ingested higher levels of simple carbohydrates as well, consistent with the model proposed above.

Stability and plasticity in the epigenetic signature of resource base

DNA methylation marks are largely established during development and subsequently carried across mitotic cell divisions. Consequently, most studies of diet or resource access effects on DNA methylation have focused on exposures during development (Wolff *et al.* 1998; Sinclair & Allegrucci 2007; Khulan *et al.* 2012). These studies have observed strong effects of maternal resource constraint during pregnancy on offspring methylation levels (Heijmans *et al.* 2008; Waterland *et al.* 2010; Tobi *et al.* 2014). Furthermore, they have emphasized the precise timing of maternal resource effects, which are sometimes limited to specific trimesters (Tobi *et al.* 2009, 2014).

The results of such studies have been widely interpreted as support for a 'critical period' model of environmental epigenetic effects, where environmental insults during development are primarily responsible for downstream effects on gene regulation (Meaney & Szyf 2005; Faulk & Dolinoy 2011; Heim & Binder 2012). However, recent work has shown that DNA methylation levels are also affected by environmental conditions later in life, including adult socio-economic status in humans (McGuinness *et al.* 2012; but see Lam *et al.* 2012), experimentally manipulated social status in captive rhesus macaques (Tung *et al.* 2012) and immune response to infection in humans (Marr *et al.* 2014; Peter McErlean *et al.* 2014; Pacis *et al.* 2015). These observations of epigenetic plasticity also extend to recent studies of energy balance and diet in humans: individuals that subsisted on a high-fat diet for 1 week exhibited epigenetic changes at thousands of CpG sites compared to randomized controls (Jacobsen *et al.* 2012). Similarly,

short-term exercise interventions induce widespread changes in DNA methylation levels (Barrès *et al.* 2012; Rönn *et al.* 2013).

The current literature thus indicates that DNA methylation plays two complementary roles. In some cases, it encodes a signature of early life experience, producing stable effects on gene regulation that persist over time. In other cases, it continues to be dynamically regulated, allowing organisms to adjust their phenotypes to prevailing environmental conditions. Whereas the effects of resource availability have been largely studied in the context of the first role (Klebig *et al.* 1995; Wolff *et al.* 1998; Heijmans *et al.* 2008; Carone *et al.* 2010; Tobi *et al.* 2014), we were able to take advantage of naturally occurring male dispersal to investigate both roles (Fig. 1).

Surprisingly, our analyses revealed a lack of support for either long-term stability or global plasticity. Instead, we found that the DNA methylation patterns of switching individuals, whether originating from or immigrating into wild-feeding groups, almost universally resembled those of lifelong wild-feeding individuals (Fig. 5). Wild-feeding individuals rely on widely distributed, seasonally available foods, and consequently experience greater seasonal and year-to-year variance in resource access compared to Lodge animals. Our results thus indicate that more challenging environments, in terms of energy balance, may leave a stronger epigenetic signature than more favourable environments. This appears to hold whether exposure occurs earlier in life – consistent with long-term early life effects and developmental constraint models – or later in life (e.g., in postdispersal males), consistent with a more sustained capacity for plasticity. Our results thus dovetail with recent work on both the evolutionary significance of early life effects (Lindström 1999; Gluckman *et al.* 2005; Botero *et al.* 2015), including in the Amboseli baboons (Lea *et al.* 2015a), as well as the possibility that epigenetic marks mechanistically mediate these effects (Weaver *et al.* 2004; Lam *et al.* 2012; Tobi *et al.* 2014). However, theoretical work is needed to connect the evolution of plasticity to expectations about the epigenetic patterns associated with different levels of adaptive plasticity (Furrow & Feldman 2014).

Finally, our findings indicate an important caveat for studies in ecological epigenetics: that inferences about stability vs. plasticity may be contingent on the direction in which an environment changes. If we had only analysed male baboons that transitioned from resource abundance to resource limitation (i.e., from the Lodge to wild-feeding resource base), our results would have supported complete plasticity. On the other hand, if we had focused only on males that transitioned from resource limitation to resource abundance, our data would have supported long-term stability. Notably, the latter type of transition is the one that has been highlighted in studies of human health, which have tended to emphasize the special importance of early life exposure (Gluckman & Hanson 2004; Schulz 2010). In contrast, our findings suggest that the specific experience of resource limitation may leave an epigenetic signature that transcends any critical window of exposure, at least in our system. Future research will be needed to assess the generality of these results. Nevertheless, we believe our results as a whole emphasize the importance – already acknowledged in evolutionary and behavioural ecology more generally – of taking a life course approach to ecological epigenetics. Organisms are a product of both their current environment and their past history, and we should expect the epigenetic patterns within their cells to reflect this combination. The more interesting outstanding questions are about the negotiation of this balance, including how it evolves.

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J.T. and A.J.L. conceived and designed the study; J.T., A.J.L., J.A. and S.C.A. collected the samples; A.J.L. performed the experiments; A.J.L. and J.T. analyzed the data; J.A. and S.C.A. contributed behavioral, life history, and demographic data; J.T., S.C.A. and J.A. provided funding support; and J.T. and A.J.L. wrote the paper, with input from all authors.

Data accessibility

For this study, we drew on previously published baboon RRBS data (Lea *et al.*), as well as RRBS data we generated for this study. All sequencing data have been deposited in NCBI (project accession SRP058411). In addition, the following data are available in the Dryad database (doi:10.5061/dryad.2d80 m): tables of methylated and total read counts (i.e., the input files for our analyses in MACAU), the output files from MACAU (including *P*-values and effect size estimates for each site) and cell-type proportion data collected from blood smears.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Supplementary Materials and Methods.

Fig. S1 Flow chart describing data processing steps and main analyses.

Fig. S2 Genomic compartment annotations used in this study.

Fig. S3 Histone marks and genomic compartments associated with chromatin states.

Fig. S4 Differentially methylated sites are enriched near genes expressed in whole blood.

Fig. S5 Effect of resource base on DNA methylation levels at the pathway level.

Fig. S6 DMRs are observed in the real data set more often than expected by chance.

Fig. S7 RRBS enriches for putatively functional regions of the genome and recapitulates known patterns of DNA methylation across the genome.

Fig. S8 Power to detect differentially methylated sites increases with sample size.

Fig. S9 Magnitude of the effect of resource availability on DNA methylation levels in different genomic compartments.

Fig. S10. Cell type proportions did not significantly differ between wild-feeding and Lodge.

Fig. S11 Enrichment of resource base-associated sites is strongest near genes expressed in whole blood, compared to genes expressed in other tissues.

Table S1 Information about males that switched between resource base conditions.

Table S2 Baboon RRBS dataset sample characteristics and read mapping summary.

Table S3 Differentially methylated regions.