RESEARCH ARTICLE

DNA Methylation as a Mechanism of Nutritional Plasticity: Limited Support From Horned Beetles

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ABSTRACT Epigenetic changes to DNA, potentially heritable alterations above the sequence level, such as DNA methylation, are thought to underlie many instances of adaptive phenotypic plasticity. Our understanding of the links between epigenetic variation and adaptive phenotypic plasticity in natural populations is limited. If DNA methylation underlies adaptive responses to different nutritional environments, methylation patterns should be correlated with differences in performance across nutritional environments, and respond to changes in the environment. Additionally, genotypes that can cope with a broader range of nutritional environments are expected to have greater flexibility in methylation patterns. We tested these predictions using horned beetles (genus *Onthophagus*), which can cope with a wide range of variation in larval nutrition. We surveyed levels of methylation across several methylated loci in lab-reared beetles originating from natural populations using a methylation-specific amplified fragment length polymorphism (AFLP) analysis. For less than half the of the loci investigated, methylation level was correlated with performance, measured as adult body size attained on a given diet, in different nutritional environments, with an overall greater effect in males (the more nutritionally plastic sex) than females. Methylation levels at most sites were influenced more by genotype (iso-female line) than by environment (dung type). Only 1 site (of 12) showed a significant genotype-by-environment interaction. Taken together, our results provide modest support for the hypothesis that DNA methylation underlies nutritional plasticity, as only 8–16% of methylated sites conformed to all of our predictions. *J. Exp. Zool. (Mol. Dev. Evol.) 9999B:1–13, 2012.* © 2012 Wiley Periodicals, Inc.

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Phenotypic plasticity, the ability of a genotype to vary its phenotype across environments, allows organisms to cope with environmental variation (Levins, '68; Moran, '92; Schlichting and Pigliucci, '98). Understanding the developmental mechanism of plasticity is key to predicting the costs and limits associated with plasticity, and thus the conditions under which it may evolve (West-Eberhard, 2003; Snell-Rood et al., 2010). Epigenetic modifications, changes to DNA above the sequence level such as DNA methylation or histone acetylation, have increasingly been suggested as a mechanism underlying plasticity (Jablonka and Lamb, 2005; Gilbert and Epel, 2009). Epigenetic mechanisms are heritable across cell divisions, and potentially across generations, thus having important implications for development

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and evolution. DNA methylation in particular, has been suggested as a promising candidate that may underlie phenotypic plasticity because it is known to be responsive to environmental inputs (Morgan et al., '99; Weaver et al., 2004; Fraga et al., 2005; Cropley et al., 2006; Hager et al., 2009; Franklin et al., 2010; Gao et al., 2010; Verhoeven et al., 2010) and levels of methylation across loci often differ between individuals or populations that vary in phenotypes (Cervera et al., 2002; Salmon et al., 2008). Furthermore, manipulation of methylation has been shown to disrupt normal patterns of plasticity, such as caste formation in social insects (Kucharski et al., 2008) or reaction norms in plants (Bossdorf et al., 2010).

Because induced patterns of methylation can be heritable (Jablonka and Raz, 2009; Johannes et al., 2009), methylation can potentially explain many instances of inter-generational plasticity, where an induced phenotype in one generation persists in the next (Verhoeven et al., 2010). For instance, methylation is, among other epigenetic modifications, thought to be important in the development of metabolic syndromes and other mechanisms by which organisms anticipate future nutritional environments (Gluckman et al., 2009; Li et al., 2010). Inherited methylation patterns could also explain why traits are sometimes slow to respond to a novel or rapidly changing environment (i.e., phenotype "mismatch").

Despite the hypothesis that methylation underlies phenotypic plasticity, we are only beginning to understand how patterns of methylation may generally relate to plasticity in natural populations. We know especially little about patterns of genotype-by-environment interactions on methylation state in natural populations even though this is the raw material necessary for the evolution of phenotypic plasticity. Similarly, despite the widespread belief that methylation underlies adaptive plastic responses to alternate environments, we know remarkably little about whether differential methylation is actually associated with performance in different conditions.

This study focused on several key predictions of the hypothesis that methylation is an important mechanism of adaptive phenotypic plasticity. First, methylation patterns should correlate with differential performance (or fitness) across environments. Second, methylation state should be influenced by the environment and genotype-by-environment interactions. These first two predictions focus on methylation state as the independent and dependent variable, respectively. Finally, genotypes with flexible methylation should show consistent, high performance across environments. Past studies have disentangled genetic and environmental contributions to methylation state through analyses of population genetic structure (Cervera et al., 2002; Herrera and Bazaga, 2010; Lira-Medeiros et al., 2010), or the use of clones and recombinant inbred lines (Johannes et al., 2009). Our approach focuses on predictions and methods stemming from the plasticity literature (Vantienderen, '91; Schlichting and Pigliucci, '98) and utilizes a common garden design to test for effects of genotype and environment on methylation state (similar to Hager et al., 2009) and to relate variation in methylation state (and methylation flexibility) to performance.

Specifically, we chose to test the above predictions using a methylation-specific amplified fragment length polymorphism (AFLP) assay to survey a broad range of methylated sites without any biases towards particular candidate genes. While AFLP methods can be noisy, they allow broad surveys of loci in non-model organisms (i.e., those without a sequenced genome). Such a survey would also give us an idea of the relative proportion of methylation sites that conform to the predictions for phenotypic plasticity. As methylation serves distinct functions within and across species, from transposable element silencing, to repression of gene expression to alternative splicing via exon methylation (Jaenisch and Bird, 2003; Zemach and Zilberman, 2010; Glastad et al., 2011), a broad survey of methylated sites would allow us to quantify the relative proportion of sites that have the potential to contribute to adaptive phenotypic plasticity in response to varying environments relative to other potential functions.

We sought to test the hypothesis that methylation underlies phenotypic plasticity using horned dung beetles as a study system. Beetles in the genus *Onthophagus* are renowned for their ability to cope developmentally, physiologically, and behaviorally with nutritional variation (reviewed in Moczek, 2009). In particular, males of numerous species develop into discrete, nutritionally cued, alternative morphs. Large males, originating from high quality larval nutrition, express disproportionately long horns used in aggressive fights with rival males over access to females and provide paternal assistance during brood provisioning. In contrast, small males, originating from low quality larval nutrition, develop rudimentary horns, invest heavily in testicular development, sneak copulations, and provide little assistance to females (Emlen, '97a; Moczek and Emlen, 2000). In addition, adult mothers adaptively adjust larval dung provisioning based on dung type and quality (Moczek, '98), and developing larvae alter the timing of developmental transitions based on resource availability (Shafiei et al., 2001). Recent transcriptomic work suggested *Onthophagus* has a "complete" DNA methylation system (Choi et al., 2010), that is, all three DNA methyl-transferases are present in the genome, avoiding problems associated with studying atypical methylation systems in model species such as *Drosophila*, yeast, and *Tribolium* (Choi et al., 2010; Johnson and Tricker, 2010; Zemach et al., 2010). In this work, we survey patterns of methylation in natural populations of *Onthophagus*, and test for links between methylation and performance as well as genotype-by-environment influences on methylation.

METHODS

Overview of Design

We surveyed patterns of methylation in *Onthophagus gazella* beetles originating from natural populations. We manipulated the

nutritional environment of beetles by rearing them on either cow or horse dung. *O. gazella*, like many onthophagine species, utilizes both dung types in nature (Moczek, unpublished data), however, given the prevalence of cow pastures in areas where this species occurs, cow dung constitutes the predominant resource type (Hanski and Cambefort, '91). Cow and horse dung differ markedly in consistency and nutritional content due to the varied efficiency of hind- and fore-gut fermentation, respectively. In particular, horse dung has roughly twice the amount of carbon and organic matter than cow dung, and slightly more nitrogen (Moral et al., 2005; Holter and Scholtz, 2007). Its higher nutritional content makes it a superior resource for many dung breeding beetles such as *Onthophagus*. For example, in the congener *Onthophagus taurus*, developing larvae only require roughly half the mass of horse dung compared to cow dung to grow to the same adult body size, and mothers adjust the quantity of larval provisions accordingly (Moczek, '98). At the same time, horse dung in the field is prone to dry out far more quickly than cow dung, which significantly limits is availability as a food and breeding resource for adult beetles as both uses require a high dung moisture content (Moczek, '98). As a consequence, many onthophagine beetles flexibly utilize either cow or horse dung depending on the exact conditions at a given location and time point.

In addition to the global qualitative differences between both dung types, natural populations of developing *Onthophagus* larvae also experience substantial variation in the availability and quality *within* each dung type (Emlen, '97b; Moczek, 2002). This variation in turn is responsible for generating dramatic variation in body size at eclosion (Moczek and Emlen, '99) even in populations restricted solely to either cow dung or horse dung (Moczek, 2002). At the same time, body size at eclosion is tightly correlated with important fitness measures, such as female fecundity (Hunt and Simmons, 2000, 2002) and male fighting success (Moczek and Emlen, 2000). In the present study, we therefore used body size attained on a given diet as an estimator of performance of a given individual in a given nutritional environment.

Beetles were reared on each dung type for two generations prior to measurement of methylation state. Previous studies have found successive changes in methylation state over consecutive generations following environmental shifts (Johannes et al., 2009; Burdge et al., 2011), so we reasoned that a shift from a putatively cow-dung rich diet in the field to a horse dung diet in the laboratory may not be measureable immediately. The experiment was designed to test whether methylation patterns varied with genotype (a beetle's mother and grandmother), environment (horse or cow dung) or an interaction between the two. To quantify methylation levels, we used a methylation-specific AFLP analysis that is often used to survey methylation in non-model species (Salmon et al., 2008; Herrera and Bazaga, 2010; Lira-Medeiros et al., 2010). This method, discussed in detail below, relies on the digestion products from two restriction enzymes that differ in their sensitivity to methylation, but that cut at the same site, to infer the presence of methylated cytosines in the genome.

Study System, Animal Rearing, and Morphological Measures

The experiment, as outlined in Figure 1, assayed 96 experimental individuals obtained from 16 mothers of 4 wild-caught beetle lines (grandmothers). More specifically, *O. gazella* ($N > 50$) were obtained from populations in cow pastures near Kaneohe, Hawaii. Given the ecology of these populations (see above), and the fact that these individuals were collected from cow pastures, it is likely that the grandmothers used to found our experimental lines had been feeding on cow dung for several weeks, and likely several generations prior. Females collected in the field were allowed to produce brood balls from both cow dung and horse dung (order randomized) over a 2-week period (1-week on each dung type). For collection of brood balls from an individual female ($N = 12$ initially, of those 6 produced brood balls on each dung type), beetles were housed in individual PVC cages approximately 10 cm in diameter and 30 cm tall, packed with a 1:3 soil:sand mixture and topped with fresh dung (water was added to the horse dung to roughly equalize the moisture content).

Eggs from these wild-caught females were allowed to hatch within their brood balls and grow to adulthood at 26°C (12:12 photoperiod). This first lab-reared generation is referred to as the "F1" or "mother" generation used to generate the experimental

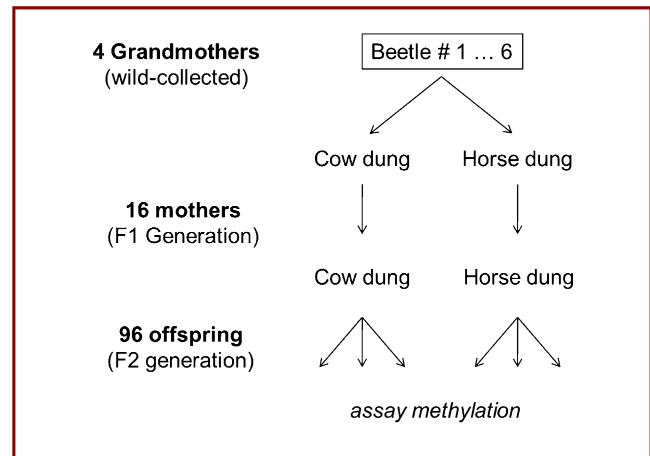

Figure 1. Overview of experimental design. Wild-collected *Onthophagus gazella* were used to found experimental lines. These grandmothers constructed brood balls on both cow and horse dung (order randomized between individuals) and beetles were reared through one whole generation on that dung type. Methylation assays were performed on just-emerged adults of the second generation in the laboratory (i.e., the grand-offspring of the wild-collected individuals).

animals (the "F2" generation). Upon emergence, the F1 generation ($N > 50$ initially; 16 mothers produced enough brood balls for further analysis) was given their larval dung type, and allowed to feed and mate (with siblings) for 2 weeks. Individual F1 females were then set up in individual PVC cages (10 cm diameter, 30 cm height) and allowed to make brood balls for 10 days on their larval dung type. The resulting eggs—the "F2" generation— were reared through to adulthood (as for the F1 generation) and sacrificed upon adult emergence. Beetles were stored in 95% ethanol at −20° C until DNA extraction.

The F2 generation consisted of 220 individuals. A subset of 96 individuals from 16 mothers of 4 lines (grandmothers collected in the field) was chosen for DNA analyses to maximize the use of 96-well plates used in each of three AFLP runs. For each mother, three males and three females were chosen that spanned both large and small sizes. We focused on body size variation as a reflection of nutritional variation. We also focused on sex differences because male *Onthophagus*, including *O. gazella*, are generally far more nutritionally sensitive than females, and other studies have found sex-specific methylation patterns in a range of taxa (e.g., *Apis*, Lyko et al., 2010). Lastly, we used body size as a measure of performance in a given nutritional environment (dung type). For each individual beetle (in the F2 generation), thorax width was measured using a dissecting microscope and Image J (NIH). We tested whether body size varied with environment using ANOVAs. All statistical tests were performed in JMP (version 8.0; SAS Institute, Cary, NC, USA).

DNA Extraction

Genomic DNA was extracted from beetle abdomens. We reasoned that shifts in methylation relevant to diet would likely occur in several tissues specific to the abdomen including the gut and fat bodies. Given the lack of knowledge of methylation in beetles, we sought to include several tissues that might experience methylation changes, but use a methylation metric that could sum variation in methylation state across tissues (see Methylation Analyses Section). While it would be informative to also investigate gene expression in other tissues such as the brain (which would be involved in taste and direct sensing of the nutritional environment), many of the relevant physiological changes associated with different nutritional diets are likely to be occurring in the abdomen.

DNA was extracted using Qiagen DNeasy kits following manufacturer's protocols (Animal Tissue: Spin Column Protocol), with only a few modifications for our tissue type. For a given individual, the abdomen was removed (using flame-sterilized scissors), placed in a 1.7 mL microcentrifuge tube and briefly frozen using liquid nitrogen. Tissue was ground (while frozen) using a sterile pestle fit to the microcentrifuge tube. To the homogenized tissue, 180 µL buffer ATL, followed by 20 µL Proteinase K, were added and the mixture incubated at 56°C for 3 hr (with hourly vortexing). Before proceeding, the sample was centrifuged to separate any remaining solid abdominal tissue or organs (e.g., the cuticle). This protocol yield on average 100 µL of 608 ng/µL concentration DNA (stored at −20°C in buffer AE until further analyses).

Methylation-Specific AFLP

We employed a methylation-specific AFLP analysis that has been commonly used to survey patterns of methylation in a range of non-model species (Salmon et al., 2008; Herrera and Bazaga, 2010; Lira-Medeiros et al., 2010). We used partially modified methods from previous studies (A. Toth, pers. commun., Vos et al., '95; Kronforst et al., 2008), described briefly here. For all primer and adapter sequences, see Supplementary Table 1 (all enzymes were obtained from New England Biolabs, Ispwich, MA). First, 500 ng of extracted DNA (in 4 µL water) was digested with 5 U *Eco*RI (100,000 U/mL) and either 5 U of MSPI (100,000 U/mL) or 5 U *Hpa*II (10,000 U/mL) in a 10 µL reaction (with 1 µL NE buffer) at 37°C for 3 hr. Second, the ligation reaction was performed by combining 3 µL of the restriction digest product with 1 µL of *Eco*RI adapter (5 pmol), 1 µL of the Msp/Hpa adapter (50 pmol), 0.05 µL T4 DNA ligase, and 1 µL ligase buffer in a 10 µL reaction held at 37°C for 3 hr and 25°C for 16 hr. The adapters were prepared by mixing the paired adapters at 10 pmol/µL (*Eco*RI) or 100 pmol/µL (Msp-Hpa), heating to 95°C for 5 min, then cooling to room temperature over a 10-min period.

We then performed two rounds of PCR on the samples. For the pre-select PCR, the ligation reaction was first diluted to 1:10 before 1 µL of template was combined with 1 µL of *Eco*R1 primer (5 µM), 1 µL of MSP/HPA primers (5 µM) and 7 µL of AFLP Amplification Core mix (AFLP Plant Mapping Kit; Applied BioSystems, Carlsbad, CA). This reaction was run at the following PCR settings: 72°C for 2 min followed by 30 cycles of 94°C for 20 sec, 56°C for 30 sec and 72°C for 2 min, ending with 72°C for 2 min and 60°C for 30 min. For the select PCR, the preselect PCR products were first diluted 1:10 before 1 µL of this template was added to 1 µL of *Eco*R1 FAM-A primer (1 µM), 1 µL of Msp/Hpa select primer mix (10 µM) and 7 µL of AFLP Core Mix. Three different select primer sets were used (Supplementary Table 1). This reaction was run at a similar PCR program except that the cycles started at a 66°C annealing temperature and dropped 1° each cycle to 56°C by the 10th cycle (melting at 94°C for 30 sec and extending at 72°C for 1 min).

For analysis, 3 µL of PCR product were combined with 6.6 µL of HiDi Formamide (Applied BioSystems) and 0.4 µL GeneScan-500 LIZ Size Standard (Applied BioSystems). Plates were heated at 95°C for 5 min, immediately chilled on ice, and then read using an ABI3730.

Methylation Analyses

Fragment analysis was performed using GeneMapper Software (version 4.0) and several subsequent data processing steps. Given that AFLP-based methods can be noisy and subjective

(determining the presence or absence of a peak), especially because overall intensity can vary between samples, individuals or plates, we developed experiment-specific analysis methods to ensure robust and replicable results (Bonin et al., 2007). First, we focused on fragments that were significantly different in size from adjacent fragments (determined using a *t*-test on GeneMapper output; for variable sites with at least 10 observations, average $P = 2.5 \times 10^{-6}$). This step eliminated any unreliable fragment classifications by the program. Second, we calculated a "methylation metric" for every variable site identified by the program. For a given site, methylation was measured as the peak height for the Msp digest, corrected for overall Msp sample intensity (the sum of all peak heights) minus the peak height for the Hpa digest, corrected for overall Hpa sample intensity. Because our samples pooled several tissues, such a continuous metric of methylation (versus the more standard discrete, "presence/absence" metric) allowed us to accommodate the fact that methylation is likely to vary across tissues (Fraga et al., 2005; Yang et al., 2011). For example, if a site was methylated in one tissue but not in an adjacent tissue, a discrete measure of methylation would classify this site as "methylated," while a continuous measure would classify this site as only partially methylated. Third, we tested whether a site was consistently methylated, as opposed to noise in the AFLP analysis producing spurious Msp-specific bands in only a few samples by testing whether the methylation metric was significantly positively skewed. We reasoned that a robustly methylated site (at the internal C site of the CCGG site) should show methylation metrics >0 while an unmethylated site would be as likely to show a negative as positive methylation metric. We focused on sites with measurements for at least 25% of individuals. Because lines differed genetically, some sites were entirely absent from certain individuals (whether they were methylated or not). Presumably the noise associated with AFLP analyses, and variation in amplification (especially for larger fragments), may have also resulted in some individuals with no measurement (peak intensity) for a given site (fragment size). To avoid making erroneous conclusions based on such "false negatives," for an analysis of a given methylated site, individuals without a measurement were not included in the analysis.

Statistical Analyses

For each reliably methylated site, we tested whether mean methylation state was related to performance (body size) in different environments and whether genotype and environment influenced mean methylation. The first set of ANOVAs performed (prediction 1) related methylation level, environment (dung type), sex, and all possible interactions (as independent variables) to performance (body size, dependent variable), run separately for each methylated site. The second set of ANOVAs performed (prediction 2) related mother, grandmother, environment, and genotype (grandmother) by environment (as independent variables) to methylation level (as the dependent variable), for each methylated site. For these analyses, mother was nested within grandmother and dung type. For each set of ANOVAs, we corrected for multiple tests by using a Bonferroni correction. We adjusted our alpha value to correct for the number of tests of interest we were performing (i.e., those that included a predicted variable such as methylation or environment). For example, if we predicted an effect of methylation state on performance in a model that also considered environment and sex and all interactions, the corrected alpha was a function of 4 variables of interest (methylation, "methylation $\times$ sex," "methylation $\times$ environment," and "methylation $\times$ sex $\times$ environment") and the 12 sites (i.e., $\alpha = 0.05/(12 \times 4) = 0.001$). Application of less stringent Bonferroni corrections, such as the sequential Holm–Bonferroni correction, did not change reported results. When significant interactions were detected (with or without the Bonferroni correction), we analyzed those relationships in more detail. In particular, for sites where methylation level had significant effects on performance (body size) that varied with sex or environment, we analyzed the effects of methylation on performance separately for each sex and environment. In addition, for sites with significant genotype-by-environment effects on methylation levels, we investigated whether G $\times$ E patterns in methylation were concordant with G $\times$ E patterns in performance (prediction 3).

RESULTS

Performance Varies With Nutritional Environment

An ANOVA that included effects of dung type, mother, grandmother, sex and a dung by grandmother interaction as independent variables revealed that beetles performed better on horse dung than cow dung ($F_{1,181} = 8.96$, $P = 0.003$; Supplementary Fig. 1), consistent with other studies (Moczek, '98). There was a marginally significant effect of an individual's mother ($F_{11,181} = 1.81$, $P = 0.06$) and no effect of grandmother ($F_{3,181} = 1.21$, $P = 0.31$). However, there was a significant interaction between grandmother and dung type ($F_{3,181} = 3.65$, $P = 0.01$, Fig. 3A) on performance (body size). In addition, there was an effect of sex on our measure of body size (thorax width; $F_{1,181} = 65.1$, $P < 0.0001$), indicating sexual size dimorphism.

Identification of Methylated Sites

We identified 12 reliably methylated sites, that is, sites with a peak difference between the Msp and Hpa digests that was significantly positively skewed, suggesting methylation (see Methods Section and Supplementary Table 2). We confirmed repeatability of a subset of the sites: replicated sites were significantly correlated, although with considerable noise as is often the case with AFLP data (Supplementary Table 3). We then tested whether methylation state was correlated across each of the 12 reliably methylated sites: for 66 possible correlations, only 6 were significant with an alpha of 0.01 (Supplementary Table 4). These analyses suggest that

methylation states are somewhat, but not entirely, independent among loci. Consequently, we analyzed sites individually.

Methylation Is Correlated With Performance in Different Nutritional Environments

We first tested the prediction that methylation state (as the independent variable) was related to performance (as the dependent variable) in different nutritional environments. We performed ANOVAs for each methylated site, testing for the effects of sex, environment, methylation status, and all possible interactions on performance (body size). Methylation was related to performance (body size) for 6 of the 12 sites (Table 1), although the correlation between methylation and body size depended on sex and environment. Correcting for multiple tests (4 "methylation effects" and 12 sites, $\alpha = 0.001$), none of these relationships remained significant (lowest $P = 0.0017$ for site 2–67).

To explore the interactions between methylation, sex and environment in more detail, we tested for correlations between methylation state and body size for each sex–environment group for each site with a significant methylation effect (P-values in Table 1 not corrected for multiple tests). Methylation was significantly correlated with performance for males on cow dung (4 of 6 sites) and females on horse dung (1 of 6 sites, Table 2). Correcting for multiple comparisons (24 possible tests, $\alpha = 0.002$), only two sites were significantly related to performance (sites 2–67 and 3–79; Fig. 2).

Methylation Is Primarily Influenced by Genotype and Secondarily by Genotype–Environment Interactions

Second, we tested the prediction that methylation state (as the dependent variable) was influenced by environment and/or genotype-by-environment interactions. Individual ANOVAs for each methylation site revealed that 7 of the 12 sites were significantly influenced by an individual's genotype—their mother or grandmother. Only two sites had significant effects of environment (Table 3); one site had an effect of environment alone, while one site had a significant genotype-by-environment interaction. The site with a significant environment effect (site 3–79; $P = 0.05$), had higher methylation levels in the horse environment relative to the cow environment [mean (SE): cow: 0.0036 (0.003), horse: 0.011 (0.003)]. However, neither of these two sites had a significant environment effect after correcting for multiple tests (24 possible tests with an environment effect, $\alpha = 0.002$).

Finally, we investigated the prediction that genotypes that coped best with nutritional variation would have the highest flexibility in methylation. We identified one site that showed significant genotype-by-environment effects on methylation (site 2–67; $P = 0.04$). We inspected genotype patterns of methylation at this site with respect to genotype-level variation in performance (body size, Fig. 3). Patterns of methylation paralleled patterns of performance between environments: for a given nutritional environment, genotypes with greater performance exhibited higher methylation levels. However, when the genotype that performed best across *both* environments (the purple genotype in Fig. 3) was contrasted to the genotype that exhibited the greatest environment-specific performance (the red genotype in Fig. 3, which performed well on horse dung but poorly on cow dung), there was no support for the prediction that methylation levels of the most nutritionally plastic genotype were more flexible (Fig. 3).

Table 1. Methylation state and performance.

Site	Sex	Enviro.	Methyl.	S × E	M × S	M × E	M × E × S
2–21	$F_{1,75} = 19.8^{***}$	$F_{1,75} = 3.64$	$F_{1,75} = 0.19$	$F_{1,75} = 0.00$	$F_{1,75} = 3.91^{**}$	$F_{1,75} = 0.94$	$F_{1,75} = 0.59$
2–67	$F_{1,72} = 24.1^{***}$	$F_{1,72} = 3.72^{**}$	$F_{1,72} = 1.82$	$F_{1,72} = 0.05$	$F_{1,72} = 10.6^{***}$	$F_{1,72} = 3.15$	$F_{1,72} = 3.31$
2–133	$F_{1,64} = 18.1^{***}$	$F_{1,64} = 0.98$	$F_{1,64} = 1.21$	$F_{1,64} = 0.33$	$F_{1,64} = 0.05$	$F_{1,64} = 0.14$	$F_{1,64} = 0.08$
2–177	$F_{1,37} = 2.37$	$F_{1,37} = 1.34$	$F_{1,37} = 4.89^{**}$	$F_{1,37} = 1.88$	$F_{1,37} = 1.52$	$F_{1,37} = 0.96$	$F_{1,37} = 4.18^{**}$
3–4	$F_{1,48} = 12.9^{***}$	$F_{1,48} = 0.63$	$F_{1,48} = 0.19$	$F_{1,48} = 0.02$	$F_{1,48} = 0.06$	$F_{1,48} = 0.12$	$F_{1,48} = 0.21$
3–79	$F_{1,61} = 16.7^{***}$	$F_{1,61} = 1.18$	$F_{1,61} = 0.71$	$F_{1,61} = 0.15$	$F_{1,61} = 3.75$	$F_{1,61} = 4.67^{**}$	$F_{1,61} = 0.33$
3–112	$F_{1,27} = 15.2^{***}$	$F_{1,27} = 0.19$	$F_{1,27} = 0.24$	$F_{1,27} = 0.007$	$F_{1,27} = 2.31$	$F_{1,27} = 4.92^{**}$	$F_{1,27} = 0.02$
3–153	$F_{1,46} = 15.0^{***}$	$F_{1,46} = 1.08$	$F_{1,46} = 0.56$	$F_{1,46} = 0.37$	$F_{1,46} = 0.001$	$F_{1,46} = 0.61$	$F_{1,46} = 0.05$
1–32	$F_{1,54} = 18.6^{***}$	$F_{1,54} = 3.83$	$F_{1,54} = 0.57$	$F_{1,54} = 0.32$	$F_{1,54} = 0.76$	$F_{1,54} = 0.10$	$F_{1,54} = 0.17$
1–51	$F_{1,76} = 17.1^{***}$	$F_{1,76} = 3.11$	$F_{1,76} = 0.003$	$F_{1,76} = 0.09$	$F_{1,76} = 0.29$	$F_{1,76} = 0.05$	$F_{1,76} = 0.83$
1–97	$F_{1,56} = 7.93^{***}$	$F_{1,56} = 2.46$	$F_{1,56} = 3.94$	$F_{1,56} = 0.002$	$F_{1,56} = 1.06$	$F_{1,56} = 2.39$	$F_{1,56} = 4.73^{**}$
1–102	$F_{1,56} = 14.1^{***}$	$F_{1,56} = 2.15$	$F_{1,56} = 1.83$	$F_{1,56} = 0.43$	$F_{1,56} = 0.01$	$F_{1,56} = 3.02$	$F_{1,56} = 2.92$

An ANOVA was performed for each of 12 reliably methylated sites, testing for their effects on body size (thorax width; dependent variable). Shown are F values for each of seven independent variables, including sex, environment (dung type), methylation state, and every interaction. Significance is indicated with asterisks—P-values in this table are *not* corrected for multiple tests—a Bonferroni correction for effects of methylation showed that none of these relationships remained significant after correcting for multiple tests.
$^{**}P < 0.05$. $^{***}P < 0.01$.

Table 2. Variable correlations between methylation and performance with sex and environment.

	Female		Male	
	Cow	Horse	Cow	Horse
2–21	$F_{1,17} = 0.01$	$F_{1,19} = 1.68$	$F_{1,17} = 4.22$	$F_{1,22} = 1.74$
2–67	$F_{1,17} = 0.17$	$F_{1,18} = 15.3^{***}$ (−)	$F_{1,17} = 1.41$	$F_{1,20} = 1.11$
2–177	$F_{1,5} = 0.00$	$F_{1,10} = 0.57$	$F_{1,9} = 6.83^{**}$ (−)	$F_{1,13} = 0.31$
3–79	$F_{1,13} = 0.50$	$F_{1,14} = 1.43$	$F_{1,17} = 16.2^{***}$ (+)	$F_{1,17} = 0.62$
3–112	$F_{1,7} = 0.87$	$F_{1,8} = 0.84$	$F_{1,5} = 7.35^{**}$ (−)	$F_{1,7} = 0.18$
1–97	$F_{1,13} = 0.02$	$F_{1,12} = 1.26$	$F_{1,13} = 8.16^{***}$ (−)	$F_{1,18} = 0.25$

For each methylated site with a significant sex- or environment-specific effect on performance (see Table 1), we performed individual ANOVAs for each sex within each dung environment. Shown are F values from these tests, with significance indicated with asterisks and the direction of the relationship indicated in parentheses. P values in this table are *not* corrected for multiple tests—a Bonferroni correction for effects of methylation showed that only two of these relationships remained significant after correcting for multiple tests (2–67 and 3–79). $^{**}P < 0.05$.
$^{***}P < 0.01$.

DISCUSSION

Methylation as a Mechanism Underlying Nutritional Plasticity

Taken together, our results provide only limited support for the hypothesis that DNA methylation underlies phenotypic plasticity in response to variable nutritional environments. Instead, our data suggest that methylation states at the majority of methylated sites identified are unrelated to adaptive phenotypic plasticity, at least for the environments we focused on. Recent work has suggested that methylation may underlie much adaptive nutritional

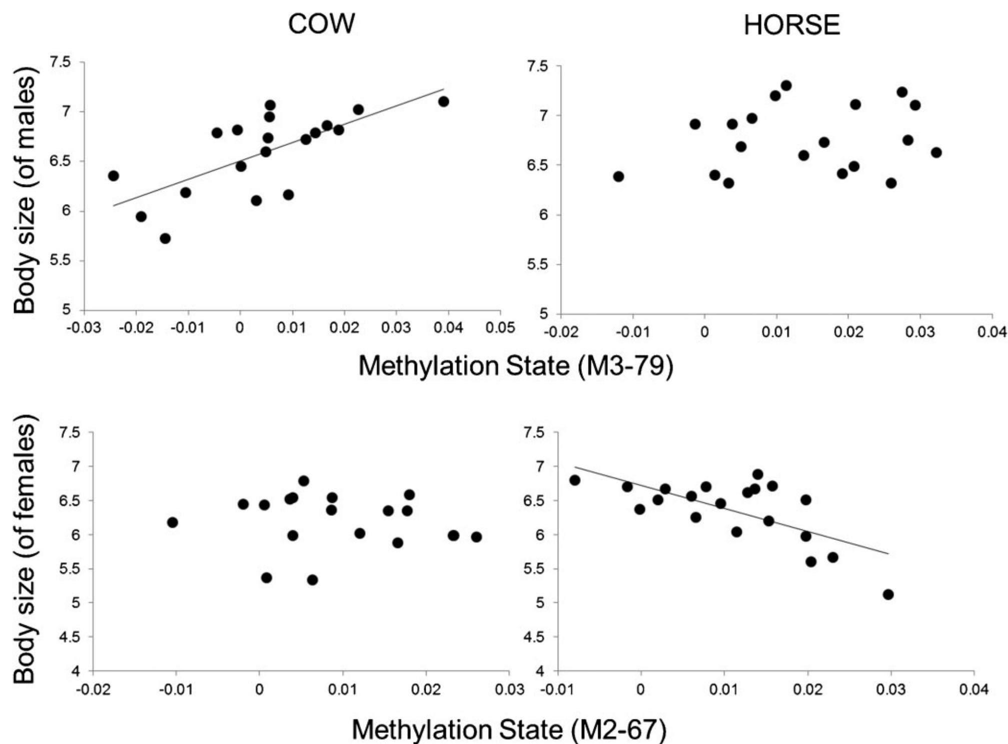

Figure 2. Performance is related to methylation state. Shown are individual ANOVAs for two methylation sites, relating methylation state to performance (body size) in one of two environments (cow or horse dung). This relationship is specific to sex and environment (see Table 2).

Table 3. Influence of genotype on methylation state.

Site	Mother	Grandmother	Environment	G × E
2–21	$F_{6,69} = 3.08^{***}$	$F_{3,69} = 0.71$	$F_{1,69} = 2.49$	$F_{3,69} = 0.56$
2–67	$F_{6,66} = 0.41$	$F_{3,66} = 2.16$	$F_{1,66} = 0.01$	$F_{3,66} = 2.86^{**}$
2–133	$F_{6,58} = 7.77^{***}$	$F_{3,58} = 5.83^{***}$	$F_{1,58} = 0.00$	$F_{3,58} = 1.73$
2–177	$F_{5,32} = 0.44$	$F_{3,32} = 7.99^{***}$	$F_{1,32} = 0.40$	$F_{3,32} = 1.15$
3–4	$F_{5,43} = 0.95$	$F_{3,43} = 3.34^{**}$	$F_{1,43} = 0.45$	$F_{3,43} = 0.37$
3–79	$F_{6,55} = 1.72$	$F_{3,55} = 0.87$	$F_{1,55} = 3.99^{**}$	$F_{3,55} = 1.48$
3–112	$F_{4,25} = 0.69$	$F_{2,25} = 4.83^{**}$	$F_{1,25} = 1.99$	$F_{2,25} = 0.78$
3–153	$F_{5,41} = 4.36^{***}$	$F_{3,41} = 3.23^{**}$	$F_{1,41} = 0.12$	$F_{3,41} = 1.96$
1–32	$F_{6,48} = 0.11$	$F_{2,48} = 0.18$	$F_{1,48} = 0.43$	$F_{2,48} = 0.23$
1–51	$F_{6,70} = 1.78$	$F_{3,70} = 1.05$	$F_{1,70} = 1.52$	$F_{3,70} = 1.38$
1–97	$F_{6,50} = 1.86$	$F_{3,50} = 3.15^{**}$	$F_{1,50} = 0.24$	$F_{3,50} = 2.32$
1–102	$F_{5,47} = 2.21$	$F_{2,47} = 1.61$	$F_{1,47} = 0.46$	$F_{2,47} = 2.91$

ANOVAs were performed testing for the effects of an individual's mother (nested within environment and grandmother), grandmother, environment (dung type) and grandmother by environment interactions (independent variables) on an individual's methylation state (dependent variable) for each of 12 sites. Shown are *F* values, with significance indicated with asterisks. *P* values in this table are *not* corrected for multiple tests—a Bonferroni correction for effects of environment showed that none of these relationships remained significant after correcting for multiple tests. $^{**}P < 0.05$.
$^{***}P < 0.01$.

plasticity (Gilbert and Epel, 2009). The present study provides critical tests in natural populations to begin to address this hypothesis. Our findings suggest that we must keep in mind that the functional roles of methylation likely vary widely, as discussed below.

We identified several reliably methylated sites in *O. gazella* originating from the wild. Consistent with other studies in insects (Glastad et al., 2011), we estimated that overall methylation levels were low: approximately 3.5% of variable AFLP fragments were reliably methylated. This estimate is likely conservative given our multiple levels of data filtering. Regardless, this finding recalls the observation that methylation levels in insects may be substantially lower than in mammals (Lyko et al., 2010) and plants, where similar methods have identified up to 30–50% of sites as methylated (Cervera et al., 2002; Herrera and Bazaga, 2010).

We tested several predictions of the hypothesis that methylation may underlie nutritional plasticity: (a) methylation patterns should be correlated with variation in performance among individuals in different nutritional environments, (b) methylation

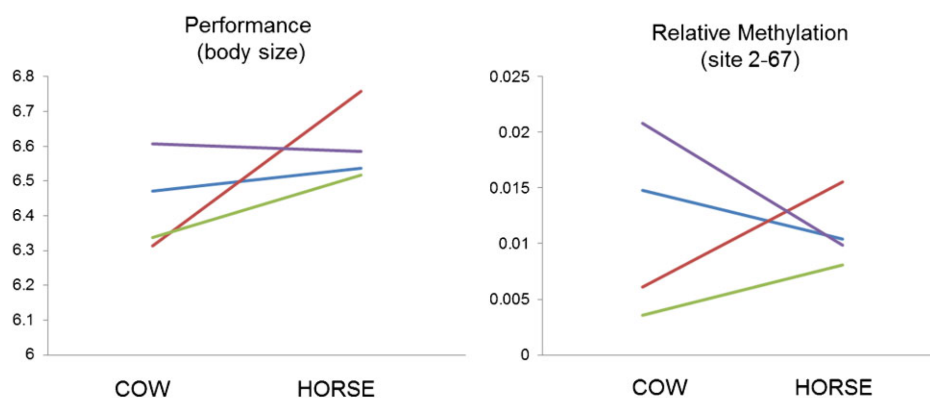

Figure 3. G × E in performance and methylation state. Beetle families showed significant genotype-by-environment interactions in performance (left panel) and overall patterns of methylation (right panel). Shown are least square means from models that include genotype (mother and grandmother), environment and grandmother by environment interactions (see Table 3). Each line represents the mean body size of offspring of a grandmother, reared on either cow or horse dung for two generations (see Fig. 1 for design overview).

levels should vary with rearing environment, and (c) genetic variation in methylation flexibility should correlate with genetic variation in nutritional plasticity.

Influence of Methylation on Performance. We first addressed whether methylation is linked to differential performance across nutritional environments. More specifically, we tested whether methylation state (as the independent variable) was correlated with a measure of performance (body size) in our two environments (dung types). We contrasted two dung types (cow and horse) that differ in overall nutritional content (Moral et al., 2005; Holter and Scholtz, 2007) and have subsequent effects on overall beetle growth (Supplementary Fig. 1, Moczek, '98). Analyses of individual sites revealed that for half of the methylated sites, methylation state was related to performance, but in a sex- and environment-specific way (Fig. 2, Table 1; not corrected for multiple tests). Methylation state was most often linked to performance on cow dung for males (4 of 6 sites, Table 2), but was also linked to female performance on horse dung (1 of 6 sites). However, after correcting for multiple tests, only two of these sites showed significant sex- and environment-specific links between methylation levels and performance.

The mechanisms underlying sex- and environment-specific links between methylation and performance are unclear. *Onthophagus* males are generally more sensitive to nutritional variation than females (Emlen, '97a; Moczek and Emlen, 2000), altering their morphology and behavior with body size (a function of larval nutrition), suggesting that selection on nutritional plasticity may be stronger in males. It is conceivable that this disparity may be heightened in particularly demanding nutritional environments, such as the use of cow dung compared to the relatively more nutritious horse dung. It is important to note that we related methylation to performance assuming that a given methylation state might influence performance in a given environment. However, we cannot distinguish against the alternate hypothesis that differences in performance have driven differences in methylation. Direct manipulations of methylation could assess this idea in the future.

Although these correlative results are suggestive of a link between performance and methylation, correcting for multiple comparisons revealed significant links between performance and methylation for only 2 of the 12 sites (Fig. 2, Table 2). Taken together, these results suggest that possible links between methylation and performance vary substantially with the specific methylation site, sex, and environment.

Environmental Influences on Methylation. If methylation underlies nutritional plasticity, we additionally predicted that methylation state would be influenced by environment. In other words, the methylation phenotype, as the dependent variable, should be influenced by the environment and/or genotype-by-environment interactions. Individual analyses of each site revealed that while genotype influenced methylation state for over half of the sites, environment was significantly important for only two sites (Table 3). Thus, we found only limited support for our second prediction, especially given that the effect for both of these environment-sensitive sites did not remain significant after correcting for multiple tests.

Our observation that genotype is an important determinant of methylation status is consistent with observations in other systems (Silva and White, '88; Sandovici et al., 2003; Kadota et al., 2007). As is often the case, it is difficult to determine whether methylation state is directly inherited or whether the propensity for a site to be methylated in an individual is determined by an allele elsewhere in the genome. In animals, methylation state is generally re-set in early development (Santos et al., 2002), yet recent work suggests that this erasure is often incomplete at certain loci (Rassoulzadegan et al., 2006; Hitchins et al., 2007), suggesting either scenario is possible. A promising future approach would be the use of clonal species (e.g., aphids, *Daphnia*) to completely control for genetic variation and isolate environmental effects, similar to human twin and clonal plant studies (Fraga et al., 2005; Verhoeven et al., 2010). Our analysis shows that the characteristics of individual methylated sites vary broadly, some being environmentally responsive, while most being relatively invariant with respect to the varying environments we focused on (dung type). It is also possible that genotypic differences were induced by environmental variation generations early and subsequently inherited by our lines.

Lastly, we predicted that genetic variation in methylation flexibility would be correlated with genetic variation in nutritional plasticity. Only one of the 12 sites showed a significant genotype-by-environment ($G \times E$) interaction. Interestingly, the $G \times E$ pattern in methylation paralleled the genetic variation in performance in the two environments (Fig. 3)—for a given environment, genotypes with high performance had high methylation levels at that site. However, the genotype with the greatest specialization for a particular nutritional environment did not show the least flexible patterns of methylation, suggesting limited overall support for this prediction. It is important to note that we only compared four lines split between two environments. Our methylation survey design (96 individuals, 3 primer sets) precluded a large number of different lines. Thus, a larger survey of genotypes (for instance, using first-generation individuals or only one sex per line) will be needed to more thoroughly evaluate the third prediction linking nutritional plasticity to methylation plasticity.

We had no a priori expectations in regards to the directionality linking variation in methylation to variation in performance. Other studies have shown that stressful environments can be associated with *either* hypo- or hyper-methylation (Yauk et al., 2008; Lira-Medeiros et al., 2010), making predictions about the directionality of changes unclear. Indeed, the directionality of

individual-performance relationships did not align with the observation of methylation patterns at the genotype and environment level. For instance, one site was positively related to individual-level performance on cow dung, but was overall higher in individuals reared on horse dung (sites 3–79; Fig. 2, Table 3), while another site was negatively related to individual performance on horse dung, but was at its highest levels in *genotypes* that did well on horse dung (sites 2–67; Figs. 2 and 3). It is clear that future work will have to address the causal links between changes in methylation and changes in performance. This will be facilitated by investigating methylation in genes with known functions as opposed to using general assay methods such as MAFLP that also come with a lot of noise.

Conclusions, Caveats, and Future Directions

Our results provide only limited support for the idea that DNA methylation may serve as a mechanism underlying phenotypic plasticity, co-opted from its presumed ancestral role in transposon silencing (Bestor, '90; Gilbert and Epel, 2009). Two sites (of 12) were linked to performance at the individual level and also varied with the environment. However, most of the surveyed sites were only influenced by genotype and did not conform to our predictions regarding methylation and nutritional plasticity. Indeed, the two sites that did conform to our predictions did not align in directionality between the analyses linking methylation and performance and those linking the environment to methylation status.

Across all analyses conducted in the context of this study, including corrections for multiple comparisons, our results suggest that only 8–16% of sites conformed to the predictions of the hypothesis that methylation underlies adaptive phenotypic plasticity. Are these sufficient levels to support the hypothesis? Most likely "yes" since our estimate of sites conforming to predictions of the phenotypic plasticity hypothesis likely represent an underestimate for several reasons. First, our criteria for inclusion of sites were stringent in order to reduce the noise inherent in AFLP studies and minimize the occurrence of false positives. This stringency gives us confidence in our results, especially so for methylated sites that conform to more than one of our three predictions. This stringency also raises the possibility that we may have missed many sites, which based on our methods, exhibited less detectable yet nevertheless developmentally relevant methylation patterns. Second, it is possible that tissue-specific variation in methylation obscured the importance of certain methylated sites, for instance sites methylated in the gut, but not other tissues in the abdomen. Third, we focused on only one developmental stage, 24 hr after emergence as adults. Given that methylation state may vary over development, it is possible that we missed important methylated sites relevant for the larval stage or later adulthood. Forth, as mentioned above, we cannot exclude the possibility that the ubiquitous genotypic variation in methylation may have been induced in previous generations by some other environmental variable, such as variable nutrition or climate experienced in the field. These considerations suggest the possibility that our results may have identified only a small portion of what in reality is a large number of methylated sites whose methylation status enables complex, diet-induced responses in development.

At the same time, it is important to consider that methylation has diverse functions within and across species, from transposable element silencing in many lineages (although not insects, Matzke et al., 2000; Zemach and Zilberman, 2010) and regulation of gene expression through promotor methylation (Jaenisch and Bird, 2003) to possibly providing between-cell selectable variation in development (Feinberg and Irizarry, 2010). In insects, methylation of gene regions, especially exons, has been suggested to play a major role in alternative splicing (Glastad et al., 2011; Lyko and Maleszka, 2011). It is possible that many methylated sites are in fact insensitive to the environment and irrelevant to differential performance in different nutritional environments. These considerations suggest that many of the methylated sites identified in our study may exhibit a given methylation status for reasons unrelated to developmental plasticity. This view is supported by our finding that only two methylation sites out of 12 could be linked to performance at the individual level and also varied with the environment.

Despite the limited number of sites that conformed to our predictions, the present study adds important information to the growing literature on the epigenetics of adaptive nutritional plasticity. For instance, previous research in honeybees suggested that methylation may underlie the phenotypic differences between queen and worker honeybees (Kucharski et al., 2008; Lyko et al., 2010). This work suggests that methylation has the potential to also play a minor role in nutritional plasticity in non-social species. Moreover, our results contribute to the growing number of studies documenting genotype-by-environment effects on epigenetic state (Hager et al., 2009; Gao et al., 2010). Lastly, our results suggest the existence of standing genetic variation for plasticity at some methylated sites, a critical prerequisite for the evolution of phenotypic plasticity via differential methylation.

However, this work also highlights the many remaining unknowns and exciting future research directions in the study of the epigenetics of plasticity. First, we know little about the nature and degree of variation in the flexibility of methylation across generations. Previous work has suggested that the epigenetic reprogramming of a cell in a new environment can occur slowly over several generations (Johannes et al., 2009). It is possible that our assessment of environmental sensitivity of a locus may have been different if we had assayed the third or fourth generation after an environmental shift. Indeed, the adaptive nature of heritable epigenetic modifications depends on the "memory" of a site relative to the rate of environmental change (Lachmann and Jablonka, '96; Pal, '98). It would be informative to

contrast flexibility in methylation across species and loci, with reference to differences in environmental variation.

Second, this and other studies on the epigenetics of plasticity focus on CG-DNA methylation. However, a quarter of methylated DNA sites in humans are non-CG sites (Lister et al., 2009). Furthermore, several additional epigenetic mechanisms exist with the potential to regulate nutritional plasticity, such as histone methylation or acetylation. Uncovering and contrasting the relative contributions of different epigenetic mechanisms to phenotypic plasticity represents one of the most exciting areas of future research in this field.

Third, we assumed that our measures of methylated sites originated in beetle DNA. However, it is possible that we are detecting methylation of symbionts in the gut system of these beetles. Emerging work suggests that *Onthophagus* beetles are associated with a range of bacterial symbionts (Feindler et al., 2011). Although adenine methylation is common in bacteria (Casadesus and Low, 2006), cytosine methylation does occur in some groups, making it possible that we are detecting symbiont rather than host methylation (Walsh and Xu, 2006). Regardless, either mechanism is evolutionarily highly interesting. In fact, the evolutionary implications of symbiont methylation may be even greater (Gilbert et al., 2010) because acquired methylation of symbionts could be more easily vertically transmitted than an acquired somatic methylation in the beetle genome, given that the latter must be transmitted to the germ cells in order to be heritable.

In conclusion, this research provides limited support for the hypothesis that methylation may underlie adaptive nutritional plasticity in natural populations. Although the majority of methylated sites did not conform to predictions, we did find evidence for links between methylation and performance, and genotype-by-environment interactions on methylation levels. The epigenetics of phenotypic plasticity is an exciting and open field.

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