**Replicated DNA methylation differences between stick insect ecotypes**

**Authors:** Clarissa F. de Carvalho1,†\*, Jon Slate1, Romain Villoutreix2, Víctor Soria-Carrasco3, Rüdiger Riesch2,4, Jeffrey L. Feder5, Zachariah Gompert6, Patrik Nosil1,2,\*

**Affiliations:**

*1School of Biosciences, University of Sheffield; Sheffield, S10 2TN, UK*

*2CEFE, Univ Montpellier, CNRS, EPHE, IRD; Montpellier, France*

*3John Innes Centre; Norwich, NR4 7UH, UK*

*4Department of Biological Sciences, Centre for Ecology, Evolution and Behaviour, Royal Holloway University of London; Egham, TW20 0EX, UK.*

*5Department of Biology, Notre Dame University; Indiana, South Bend, 11111, USA*

*6Department of Biology, Utah State University; Logan, Utah, 84322, USA*

†*Present address: Departamento de Ecologia e Biologia Evolutiva, UNIFESP; Diadema, 09972-270, Brazil*

***\*Correspondence:*** [**clarissa.carvalho@unifesp.br**](mailto:clarissa.carvalho@unifesp.br)**;** [**patrik.nosil@cefe.cnrs.fr**](mailto:p.nosil@cefe.cnrs.fr)

**Abstract:**

Epigenetic mechanisms, such as DNA methylation, can influence gene regulation and affect phenotypic variation, raising the possibility that they contribute to ecological adaptation. To being to address this issue requires high-resolution sequencing studies of natural populations to pinpoint epigenetic regions of potential ecological and evolutionary significance. However, such studies are still relatively uncommon, especially in insects, and are mainly restricted to a few model organisms. Here, we characterize patterns of DNA methylation for natural populations of *Timema cristinae* adapted to two host plant species (*i.e.,* ecotypes). By integrating results from sequencing of whole transcriptomes, genomes, and methylomes, we investigate whether environmental, host, and genetic differences of these stick insects are associated with methylation levels of cytosine nucleotides in CpG context. We report an overall genome-wide methylation level for *T. cristinae* of ~14%, being enriched in gene bodies and impoverished in repetitive elements. Genome-wide DNA methylation variation was strongly positively correlated with genetic distance (relatedness), but also exhibited significant host-plant effects. Using methylome-environment association analysis, we pinpointed specific genomic regions that are differentially methylated between ecotypes, with these regions being enriched for genes with functions in membrane processes. The observed association between methylation variation with genetic relatedness and the ecologically-important variable of host plant suggest a potential role for epigenetic modification in *T. cristinae* adaptation. To substantiate such adaptive significance, future studies could test if methylation has a heritable component and the extent to which it responds to experimental manipulation in field and laboratory studies*.*

**Keywords:** DNA methylation, epigenetics, natural populations, insects, host-plant adaptation

**Introduction**

Epigenetic mechanisms are receiving increasing attention as possible contributors to phenotypic diversity, adaptation and evolution (Bossdorf *et al.*, 2008; Hu & Barrett, 2017; Richards *et al.*, 2017; Verhoeven *et al.*, 2016)⁠⁠. However, questions remain about the processes shaping epigenetic variation in natural populations, and their ecological and evolutionary consequences (Husby, 2022; McGuigan *et al.*, 2021)⁠⁠. Currently, the best-studied epigenetic mechanism is DNA methylation (hereafter ‘methylation’), which can influence gene regulation (Law & Jacobsen, 2010). For instance, methylation can affect the binding of transcription factors to promoters and can influence chromatin structure (Cedar & Bergman, 2009)⁠⁠. We, therefore, focus on methylation here.

Like other epigenetic mechanisms, methylation has potentially complex dynamics (Fig. 1). Methylation can respond to environmental stimuli, such that it mediates phenotypic plasticity (Duncan et al., 2014; Hu & Barrett, 2023). Methylation can also be influenced by the genetic background in different steps of its molecular pathway (Adrian-Kalchhauser *et al.*, 2020; Richards, 2006). For example, methylation could be completely dependent on the genotype (Dubin *et al.*, 2015)⁠, loosely potentiated by the genetic background (Morgan *et al.*, 1999), or largely independent of it (*i.e.* methylation marks that are environmentally-induced or resultant from processing errors, both often referred to as *‘*pure’ epigenetic variation; (Cubas *et al.*, 1999; Yao *et al.*, 2021)⁠. Methylation patterns have been shown to be transmitted across generations in plants (van der Graaf *et al.*, 2015; Zhang *et al.*, 2018)⁠, vertebrates (Heckwolf *et al.*, 2020; Hu *et al.*, 2021; Kelley *et al.*, 2021)⁠, and insects (*i.e.,* bees, Yagound *et al.*, 2020). However, the relative contributions of genetic variation, environmental effects, and their interaction in shaping patterns of methylation and their transmission across generations generally remains unclear, even in the best studied model organisms (Adrian-Kalchhauser *et al.*, 2020; Anastasiadi *et al.*, 2021; Stajic & Jansen, 2021)⁠. Thus, the role of methylation in ecological and evolutionary processes, including adaptation, is still a largely open question that requires high-resolution studies of methylation in a range of organisms.

Resolving the adaptive significance of DNA methylation is a multi-staged endeavor. A critical first step can be the characterization of patterns of variation in replicated natural populations to test for possible ecological associations in the wild (Bossdorf *et al.*, 2008; Richards *et al.*, 2017). Such variation may be missed in laboratory experiments and therefore studies in nature complement laboratory approaches (Herrera *et al.*, 2014; Husby, 2022; Ledón-Rettig, 2013). Several studies in nature have now shown that methylation can vary across environmental gradients (see reviews: Hu & Barrett, 2017; Richards *et al.*, 2017; Verhoeven *et al.*, 2016)⁠, contribute to invasion potential (Hawes *et al.*, 2018)⁠, and influence host-parasite interactions (*e.g.,* Hu *et al.*, 2018; Sagonas *et al.*, 2020)⁠. However, the majority of studies of natural methylation were performed with anonymous markers or with reduced representation sequencing (rather than whole-methylome sequencing), which can limit inferences and conclusions (Lea *et al.*, 2017). Therefore, high-throughput and resolution sequencing data are required to better identify and pinpoint differentially methylated genomic regions and genes associated with environmental differences, reflecting a possible involvement with ecological adaptation. We provide such a study here for the stick insect *Timema cristinae* (study system details below).

Notably, methylation patterns and mechanisms also vary phylogenetically (Feng *et al.*, 2010; Zemach *et al.*, 2010), further complicating efforts to draw general conclusions based on comparisons from a few restricted groups. For example, DNA methylation marks tend to be concentrated in actively expressed genes in teleost fishes, but are found upstream of inactive genes in mammals (Adrian-Kalchhauser *et al.*, 2020; McGaughey *et al.*, 2014) and in birds (Lindner et al., 2021). Moreover, insects and other invertebrates exhibit lower levels of genome-wide methylation compared to vertebrates (Suzuki & Bird, 2008; but see de Mendoza *et al.*, 2019), such that whole-methylome sequencing may be critical to detect significant differences among insect populations (de Carvalho, 2023). Information concerning insects is further limited by the group’s main model genetic organism, *Drosophila melanogaster,* being depauperate of methylation (Bewick *et al.*, 2017; Zemach *et al.*, 2010)⁠. Therefore, studies that combine higher resolution epigenomic tools with relevant ecological variables in a wide diversity of systems, most notably insects, are needed to help resolve the importance of methylation to ecological and evolutionary processes, which could differ among organisms (Husby, 2022).⁠

Here, we investigate natural methylation variation in *T. cristinae* stick insects, a model for adaptation and speciation. *Timema cristinae* are wingless, plant-feeding insects native to the Santa Ynez Mountains in California (USA, Vickery, 1993). Previous studies have identified important ecological variables affecting the evolution of this species, most notably the host plant species the insect lives and feeds on, which defines two ecotypes (Nosil, 2007; Nosil & Crespi, 2006; Sandoval, 1994a, 1994b). *Timema cristinae* are primarily found on the host plants *Ceanothus spinosus* (Rhamnaceae) and *Adenostoma fasciculatum* (Roseaceae; Fig. 2). Divergent selection between these hosts contributes to ecological reproductive isolation between ecotypes, most markedly via selection on a color-pattern trait that confers crypsis against visual predators (Nosil & Crespi, 2006). Specifically, the presence versus absence of a white dorsal stripe is highly heritable and is encoded by a major locus on linkage group 8 (LG8), named *Mel-Stripe* (Nosil *et al.*, 2018)⁠. The ecotypes also differ in traits other than color-pattern, such as body size (Nosil & Crespi, 2006)⁠, host plant preference (Nosil *et al.*, 2006)⁠, mate choice (Nosil *et al.*, 2002)⁠, and cuticular hydrocarbons (CHCs) (Riesch *et al.*, 2017)⁠. Thus, host-plant is a key ecological variable that affects adaptation in this species. These host-associated differences, along with extensive background information on the evolutionary ecology of *Timema*, make the system an interesting candidate to investigate patterns of DNA methylation and its possible adaptive significance.

To accomplish this goal, we integrated whole-genome, methylome, and transcriptomic data from natural populations of *T. cristinae*. Our strategy was three-pronged. Our first objective centered on describing the methylome of *T. cristinae*. This involved characterizing general patterns of methylation across the *T. cristinae* genome, assessing how levels of methylation in *T. cristinae* compared to other insect species, and determining whether and how methylation covaried with gene expression levels in the stick insect. We then focused on testing for associations of genome-wide methylation with factors indicative of its possible role in adaptation, namely genetic variation, geographical distance, and two environmental factors: climatic variation and host-plant use. Finally, we use methylome-environment analyses to investigate the association between methylation variation and environmental differences for specific genetic regions. We did so by examining whether DNA methylation in *Timema* is associated with the replicate use of different host-plant species and if any such differentially methylated regions (DMRs) consistently displayed particular molecular functions. To our knowledge, our results represent the first population-level study to investigate whole-methylome variation in a wild species of insect.

**Methods**

***Sampling***

Our sampling strategy was designed to capture methylation variation across the patchy and heterogeneous chaparral landscape that *T. cristinae* inhabits. Here, a ‘population’ is defined as all *T. cristinae* collected within a homogeneous patch of a single host species at a given geographic locality, following previous *Timema* studies (Nosil, 2007). Key factors dictating the selection of populations for the study were: species and abundance of host plants, elevation, and the climatic and geographical distance between populations (*Supplementary Materials*).

We selected 12 populations spanning 9 geographic localities. This comprised four localities with only *Adenostoma,* two with only *Ceanothus*, and three localities where patches of the two different host species grow side-by-side (*i.e., ‘*parapatric’ populations; Fig. 2.; Table S2). Individuals from the selected 12 populations were sampled on the same date (25th April 2017). Specimens were collected using sweep nets and flash frozen using liquid nitrogen before being store at -80OC to minimize the effects of sampling on methylation.

Two similarly-sized females were selected from each population (n=24 samples in total) to perform whole-genome bisulfite sequencing (Table S3). The sample size per population used in this work is comparable to other studies using whole-genome bisulfite sequencing (*e.g.,* Gore *et al.*, 2018; Metzger & Schulte, 2018)⁠, and is further increased in downstream analyzes as the samples are grouped per ecotype.

***Whole-genome bisulfite sequencing***

Half of each specimen’s body (cut longitudinally) was used to isolate its genomic DNA using DNeasy Blood and Tissue Kits (Qiagen). Our method of DNA isolation therefore generated a mix of DNA from different tissues, and, as a result, our measures of methylation should be considered as whole body estimates. This procedure has been used in a number of other studies in insects(*e.g.,* Bain *et al.*, 2021; Glastad *et al.*, 2016; Lewis *et al.*, 2020; Yu *et al.*, 2023), and is a reasonable starting point for the first epigenetic study within a system. The samples were treated with sodium-bisulfite before being sequenced, which converts non-methylated cytosine residues into uracil, but leaves 5-methyl-cytosines unaffected (Cokus *et al.*, 2008). Thus, only the cytosines that are methylated are retained after this treatment, and the unmethylated ones are output as uracil, later amplified as thymine following polymerase chain reaction. We included non-methylated cl857 Sam7 Lambda phage DNA (Promega Corporation) as a spike-in in each sample (1% of the final volume). One *T. cristinae* sample (individual *17\_0015*) was sequenced without sodium-bisulfite treatment as a control (*i.e.,* the non-methylated cytosines in the sample were not affected). The sodium-bisulfite treatment and high-throughput sequencing were performed by the Biomedicum Functional Genomics Unit (FuGU, Helsinki). The libraries were sequenced using the Illumina NextSeq 500 platform, with High Output 2 x 150 bp runs. In total, three flow cells with four lanes were run. See *Supplementary Materials* for details regarding the bisulfite sequencing steps.

***Filtering and mapping reads and initial quality control steps***

The BS-converted raw reads were filtered using Trimmomatic 0.36 (Bolger *et al.*, 2014), and read quality assessed using FASTQC v0.11.5 (Andrews *et al.*, 2015; see *Supplementary Materials*). High quality reads were initially mapped to the BS-transformed phage DNA (GenBank-EMBL: J02459) using Bismark 0.16.1 (Krueger & Andrews, 2011)⁠, yielding a mean of 737,086 reads uniquely mapped across samples [626,125 – 848,047; 95% confidence interval, CI]. These estimates were used as a proxy to calculate the BS-conversion efficiency in each sample. We obtained amean of 0.3% of methylated cytosines followed by guanines (*i.e.,* C-phosphate-G dinucleotide; CpG hereafter) across samples, representing 99.7% conversion efficiency (Table S4). We focused on CpGs because it is the main context in which DNA methylation occurs in animals, including insects (Feng *et al.*, 2010; Zemach *et al.*, 2010).

Non-phage reads (mean 23,262,914 [23,151,953 – 23,373,875, 95% CI]) were aligned to the *T. cristinae* reference genome 1.3c2 (Nosil *et al.*, 2018), which was first BS-converted *in silico* (following Krueger & Andrews, 2011)⁠. The mapping yielded a mean of 10,232,740 [9,803,341 – 10,662,139] reads uniquely mapped (mapping efficiency of 44.0% [43.3% – 44.7%]; Table S5). In the BS-control sample (*i.e.,* individual *17\_0015*), we expected an elevated percentage of cytosines being called as methylated by Bismark, because the unmethylated ones were not affected by the treatment (*i.e.,* not transformed into thymines). Indeed, Bismark called 98.0% methylation for CpG context in this sample, implying the software was sensitive to detect cytosines in the methylated state.Methylation calling for every cytosine was performed using Bismark (Krueger & Andrews, 2011), yielding a total of 37,622,963 cytosines.

***Genetic sequencing (genotyping-by-sequencing)***

We obtained new genotyping-by-sequencing data (GBS) from the 24 individuals with sequenced methylomes to estimate genome-wide variation between individuals in the samples. We also obtained previously published GBS accessions of other *T. cristinae* specimens from the same populations studied here, when available (Comeault *et al.*, 2015; Lindtke *et al.*, 2017; Riesch *et al.*, 2017⁠; Table S6), and obtained new GBS data from populations not previously sequenced (*i.e.,* fromBT, OUT, SC, and SCN localities; Table S7) to ensure adequate sample sizes for calling single nucleotide polymorphisms (SNPs) and estimating genome-wide variation for the study populations.

To acquire new GBS data, genomic DNA was isolated using DNeasy Blood and Tissue Kits (Qiagen), and genomic libraries were prepared following Parchman *et al.* (2012), as previously implemented in *Timema* (Comeault *et al.*, 2015; Nosil *et al.*, 2012, 2018)*,* and Peterson *et al.* (2012). Reads from all data sets were processed together (following Comeault *et al.*, 2015; Nosil *et al.*, 2018; Riesch *et al.*, 2017), and mapped to the *T. cristinae* reference genome 1.3c2 (Nosil *et al.*, 2018)⁠. SNPs were called by a custom *Perl* script (Comeault *et al.*, 2014)⁠ using SAMTOOLS (Li *et al.*, 2009)*⁠* *mpileup* and BCFTOOLS (yielding 533,420 SNPs). Custom *Perl* scripts were used along with a C++ program (alleleEst 0.1b) to estimate the genotypes based on a hierarchical Bayesian model (Gompert *et al.*, 2013). Following previous studies in *Timema* (*e.g.,* Comeault *et al.*, 2015; Riesch *et al.*, 2017), we calculated the empirical Bayesian posterior probabilities for the genotypes of each locus using the genotype likelihoods estimated by BCFTOOLS along with Hardy–Weinberg priors from our estimated allele frequencies. We then computed the posterior mean genotype as a point estimate of the genotype for each individual, at each locus.

***Controlling for genetic polymorphisms (single nucleotide polymorphisms, SNPs) in methylation calls***

BS-seq data can lead to erroneous methylation calls when bisulfite transformed data (from C-to-T or G-to-A in the reverse strand) is subsequently aligned to a reference genome. This is because a natural SNP could be assigned as a confounding differently methylated position. Therefore, controlling for SNP variation is an important quality control step to confidently assess methylation levels (Lea *et al.*, 2017). Notably, this step is rarely conducted (exceptions aside, *e.g.,* (Heckwolf *et al.*, 2020; Hu *et al.*, 2021; Schmid *et al.*, 2018) and is a technical strength of our study.

To this end, we first used the BS-control sample (*17\_0015*, Table S3) to estimate potential C/T and G/A SNPs and compare these to its BS-treated equivalent. Based on this comaparison, we estimated 0.5% of the CpGs in the methylation data were in fact SNPs (*Supplementary Materials*). We then used the GBS data along with 98 previously published whole-genome sequences (Riesch *et al.*, 2017; Soria-Carrasco *et al.*, 2014)⁠ to make a list of C/T and G/A SNPs that could confound the methylation calling. From this list, 10.5% [10.4%–10.6%] of SNPs overlapped with CpG sites in the BS-treated samples(Table S8, *Supplementary Materials*)*.* These values are considerably higher than the proportion of SNPs called in the BS-control sample with its BS-treated equivalent (only 0.5%). Thus, with this latter approach, there was likely an overestimation of confounding SNPs in the methylation tables, but the approach is conservative and ensures most methylation polymorphisms (SMPs) we study are unlikely to be genetic polymorphisms (SNPs). Thus, we removed all the CpG sites in the BS-treated data that overlapped with SNPs to reduce these confounding effects.

***Description of* T. cristinae *methylome***

For each sequenced individual, methylation levels were calculated for each site as the total number of methylated cytosines (*i.e.* unconverted C) divided by the total number of reads mapped to the site. We used the annotated *T. cristinae* genome 1.3c2 from Villoutreix *et al.* (2020) to obtain information about DNA methylation patterns in genes, in both exons and introns. Only the genes with InterPro (EMBL-EBI) or Gene Ontology accessions (GO, UniProt) were selected, resulting in a total of 19,383 retained genes. Information about repetitive elements and transposons were extracted from the *T. cristinae* RepeatMasker database (which includes short and long tandem and interspersed repeats; Villoutreix *et al.*, 2020). Results are represented in Table S10. We used enrichment analyses to estimate the probability that CpGs in different regions of genes and flanking sequences were more methylated than expected by chance (*Supplementary Materials*). All these enrichment analyses were performed using R (v3.6.3) (R Core Team, 2020) (*Supplementary Materials*).

***RNA-seq analyses***

We generated RNA-seq data from 18 of the same 24 individuals that were used for methylation analysis (Table S3), with the goal of testing for a general association between gene expression and methylation. The RNA extractions, library preparations and sequencing were performed by Genome Quebec. Total RNA for each individual was extracted from the remaining half of the specimens’ bodies using the Quiacube animal tissue kit and protocol. Details about library preparation and about filtering reads are in the *Supplementary Materials.* The reads were aligned to the *T. cristinae* reference genome 1.3c2 (Nosil *et al.*, 2018)⁠ using STAR v2.7.3a (Dobin *et al.*, 2013)⁠, and sequenced read pairs were assigned to genes in the *T. cristinae* reference genome using *featureCounts* (Liao *et al.*, 2014). Variance-stabilized transformation of these data was performed using the R package *DESeq2* v1.28.1 (Love *et al.*, 2014)⁠. We ranked genes according to their mean expression across the 18 samples before being divided into 100 bins (see *Supplementary Materials* for details on *STAR* and *DESeq2* steps).

***Association between genome-wide methylation and genetic, geographical and environmental distances***

We used methylKit v1.0.0 (Akalin *et al.*, 2012) to generate a single table with methylation calls at each site for all 24 individuals (*i.e.,* SMPs;see *Supplementary Materials*). Euclidean distances in methylation levels were then estimated between each pair of the 24 sequenced individuals. Genetic distances from the 24 individuals’ GBS data were estimated using RapidNJ 2.3.0.2 (Simonsen *et al.*, 2008). This software calculates pairwise distances between individuals based on the Kimura two-parameter model (Elias & Lagergren, 2007; Kimura, 1980; Simonsen & Pedersen, 2011). We first estimated the association between genome-wide methylation distances and genetic distances using Mantel tests, performed in the *vegan* R package (Oksanen *et al.*, 2022), based on 10,000 permutations.

We then also fit Bayesian linear mixed models to estimate the factor (or combination of factors) best explaining genome-wide methylation distances between individuals (Clarke *et al.*, 2002; Gompert *et al.*, 2014)⁠, including random effects accounting for the pairwise nature of the predictor and response distance matrices. The Bayesian approach uses a Markov Chain Monte Carlo framework to estimate the regression coefficients and deviance information criterion (DIC) for model selection. The model was fit via the *rjags* R package (Plummer, 2018)⁠, including genetic, geographical, host-plant and climatic distances to explain the methylation distances between individuals. Geographical distances were calculated using the geodesic distance between coordinate points (logarithmically transformed, Rousset, 1997). Host was coded by whether a pair of insects were collected from the same (0) or different plant species (1). We obtained climate information from WorldClim database (Harris *et al.*, 2014)⁠ at each locality. We used the two main axes from a principal component analysis (PCA, PC1=66.4% and PC2=25.5%, Fig. S1, Table S1; S*upplementary Materials*) to summarize 19 bioclimatic variables. We estimated the climatic distances between individuals using Euclidean distances. We ran three parallel chains of the model, with 10,000 iterations and a burn-in of 2,000 iterations, thinning interval of 5 and 3 chains each. All statistics were performed using R (R Core Team, 2020)⁠.

***Methylome-environment association analyses using MACAU: designating DMRs***

To further investigate the association between host plant and methylation, we delimited subsets of the genome that were most strongly differentially methylated between host ecotypes (*i.e.,* differentially methylated regions, DMRs, hereafter). We did so using an approach analogous to genotype-environment association methods (GEA), which search for correlations between genetic variation and environmental variables (Forester *et al.*, 2018)⁠. Specifically, we performed a methylome-environment association analysis using binomial mixed models (MACAU: Mixed-model association for count data via data augmentation) (Lea *et al.*, 2015)⁠. Briefly, this model estimates whether a variable (predictor) is associated with methylation levels at a specific locus or region. Its binomial component handles methylation count data, estimating the level of methylation for each CpG locus based on the number of reads with methylated cytosines and the total coverage. In addition, MACAU controls for population structure by incorporating a matrix of pairwise genetic kinship (in our case, estimated from GBS genetic data) – treated as the variance-covariance matrix for the heritable component of the random effects. The kinship matrix contributes to the value of the response variable, but does not affect its non-heritable part (Lea *et al.*, 2015). This method can enhance the power to detect a potentially true association between methylation variation and the environment (Lea *et al.*, 2017). The non-heritable component consists of the variation that is due to independent environmental noise (see Lea *et al.* 2015 for details concerning MACAU).

For the MACAU analysis, we summarized the methylation data into 1 kilobasepair (kbp) non-overlapping tiling windows to increase the per region sequencing coverage, and to enhance the model’s statistical power. Coverage outliers above the 99.9th percentile, identified with methylKit v1.0.0 (Akalin *et al.*, 2012) were removed from the analysis along with SNP variants (see S*upplementary Materials* for details). We retained the regions with a minimum coverage of 10 reads per tile comparable to other studies (*e.g.,* Heckwolf *et al.*, 2020⁠), yielding 428,092 methylation tiles. Following Lea *et al.*, 2016, we excluded all methylation tiles that were consistently hypo-methylated (mean DNA methylation level < 10%) and hyper-methylated (mean DNA methylation level > 90%). We further excluded tiles in which the standard deviation of methylation among the samples was below 5% (*i.e.,* least variable regions). These steps yielded 82,696 1kbp methylation tiles (64,713 in assembled linkage groups of the reference genome, of which 73.5% of the tiles were located within genes).

We used the 1kbp methylation tiles to model the association between host plant and methylation levels. We additionally used the first two principal component axes of climatic variation as covariates along with the BS-conversion efficiency estimated for each sample (using the non-methylated Lambda phage), and flow cell batch to remove possible variation due to technical artifacts. The matrix of pairwise kinship was inferred using GBS data (Zhou *et al.*, 2013), see *Genetic sequencing* section). We used the tail of empirical distribution of *p-values* to identify DMRs differing between hosts as regions of interest for the MACAU analysis, examining the robustness of the results to different cut-off thresholds. Here, we report results based on quantiles varying from the 0.04th to the 0.4th most significant percentile of *p-value*s (which varied from 0.0004 to 0.0061) to designate DMRs. We found qualitatively similar results across different cut-offs (see *Discussion* for further elaboration). The MACAU analyses were performed with 100,000 sampling steps and burn-in of 50,000 iterations, with the filtering ratio threshold equal 1.

As a complementary approach to MACAU, we used Bayesian regressions performed using R (R Core Team, 2020)⁠ to estimate the combination of factors best explaining the methylation distances between individuals for the DMRs, following the same approach detailed above (Clarke *et al.*, 2002; Gompert *et al.*, 2014)⁠. We estimated Euclidean distances on methylation levels calculated as the total number of methylated cytosines divided by the total number of reads mapped to the 1kbp tile. We fit the model using geographical, host plant and climatic distances to explain the methylation distances in DMRs delimited by the different quantiles of the *p-value* distribution, and also genome-wide (*i.e.,* all methylation tiles used at MACAU).

# ***Gene ontology (GO) enrichment in DMRs***

To test for GO enrichment in DMRs, we used the R package TopGO v2.52.0 (Alexa & Rahnenfuhrer, 2023). For this analysis, we used the tilling windows within genes (n=47,696) and the *T. cristinae* genome annotation (v1.3c2; Villoutreix et al., 2020). The analysis was performed comparing methylated regions that were significantly different according to host plants (*i.e.,* DMRs according to different quantiles of the empirical *p-value* distribution) versus the remaining tilling windows. Fisher’s Exact Test was used to calculate the significance of the enrichment.

**Results**

***DNA methylation is enriched in the gene body and correlated with gene expression levels***

We first quantified general patterns of DNA methylation in *T. cristinae*. To begin with, we observed that the *T. cristinae* genome only possesses DNA methyltransferase 1, which maintains methylation patterns after cell division (Goll & Bestor, 2005), but does not contain DNA methyltransferase 3, that adds methylation in a d*e novo* manner (Table S9; see *Supplementary Materials* for further discussion). We next estimated that the overall methylation level of cytosine nucleotides in CpG context was around 14% in *T. cristinae*. Methylation marksmainly targeted gene bodies*,* with enriched levels in both exons and introns compared to the genomic background levels (2.4x, *P*<0.001 for both genomic features, permutation test; Fig. 3A). Methylation was not elevated for short repeats or transposable elements (TEs). If anything, TEs appeared slightly impoverished in methylation (enrichment 0.9x, *P*<0.001, permutation test*;* Table).

Gene body methylation levels tended to increase from 5’→3’ in genes (Fig. 3B), and were higher in genes with more exons (*t*=32.6, *P*<0.001; unpaired t-test; Table S11). Gene ontology enrichment analyses indicated that highly-methylated genes generally had housekeeping functions (*i.e.,* involved in basic cellular functions), while those with lower methylation levels tended to have more dynamic functions (*e.g.,* cell signaling and membrane receptors; Tables S12-S13). Finally, we found that methylation was positively correlated with levels of gene expression (Spearman rho=0.57, *P*<0.001, Fig. S2).

***Genome-wide DNA methylation varies with genetic distance and host-plant ecotype***

We next tested for associations of genome-wide methylation with genetic, geographic, host plant and climatic differences among individuals and collection sites. We found that pairwise methylation distances were mainly correlated with genetic distance between populations (*r*=0.65, *P*<0.001, Mantel test; Fig. 4). Methylation also significantly varied with geographical distance (*r*=0.33, *P*<0.001, Mantel test), which could result from the association between genetic and geographical distances (*i.e.,* isolation-by-distance, IBD, *r*=0.50, *P*<0.001, Mantel test). In contrast, genome-wide methylation distances did not vary with host-plant considered alone (*r*=0.00, *P*=0.78, Mantel test), or with climate (*r*2=0.00, *P=*0.40, Mantel test).

Multivariate analyses using Bayesian linear mixed model regression indicated that genetic and host differences together represented the best fit model explaining methylation distances, according to the lowest distance information criterion (DIC=324.8; Table S14). In this model, genetic distances had a major explanatory effect on genome-wide methylation (standardized regression coefficient: βGEN= 1.00, [0.92, 1.08; 95% equal tail probability interval, ETPI]),while host plant had a smaller but nonetheless credible effect (βHOST= -0.08, [-0.18, -0.02; 95% ETPI]).

***Differentially methylated regions across the genome associated with host plant ecotype***

To further assess the relationship between methylation and host, we conducted a second, more fine-grained analysis focused on differentially methylated gene regions (DMRs) displaying ecotype associated differences to augment the broad genome-level results presented above. Specifically, we used 1kbp tilling windows to identify and evaluate regions distinguishing the ecotypes by a methylome-environment association analysis (*i.e.,* MACAU). This model accounts for the genetic covariance among individuals (see *Methods;* Lea *et al.*, 2015)⁠ and, thus, is similar in this regard to methods commonly employed in genotype-environment association methods and genome-wide association mapping studies.

Three general patterns emerged across the range of *p-value* cutoffs used to designate the DMRs (Table S15). First, we found that the DMRs were distributed genome-wide, and, thus, were not restricted to one or a few specific linkage groups or gene regions (Fig. 5; Fig. S3). More than two-thirds of the DMRs were located within gene bodies (mean 72.0% [70.6% – 73.4%, 95% CI], Table S15). This proportion matches that of the full set of methylation tiles analyzed with MACAU (*i.e.,* 73.5%), therefore, it does not indicate a particular enrichment of DMRs within genes. Overall, a significantly greater proportion of the DMRs (mean 61.3% [59.6% – 62.9%, 95% CI]) showed higher levels of methylation for insects collected from *Adenostoma* than *Ceanothus* (1.2x, *P*<0.001, permutation test, *Supplementary Materials*; Table S15). The absolute difference in the mean methylation levels for DMRs between individuals of different ecotypes was on average 13.1% [12.8% – 13.4%, 95% CI].

Second, our results suggest that methylation distances in DMRs are better explained by host-plant ecotype than by geographical or climatic differences. Specifically, using Bayesian linear mixed models, we found that the best supported model to explain methylation variation in the different quantiles designating DMRs was a combination of geographical distance and host ecotype (Table S16). The results revealed that DMRs clearly distinguished ecotypes, showing more elevated standardized regression coefficients in all quantiles (*e.g.,* 0.04th quantile: *β*HOST*=*0.15 [0.13 – 0.16; 95% ETPI]; 0.4th quantile: *β*HOST*=*0.37 [0.35 – 0.39]; Fig. 5B-C; Fig S4). In contrast, the DMRs were only weakly associated with geographic distance (*e.g.,* 0.04th quantile: *βG*EOG*=*0.01 [0.00 – 0.02]; 0.4th quantile: *β*GEOG*=*0.02 [0.01 – 0.04]). These patterns for DMRs contrasted markedly with genome-wide trends outside DMRs, where the best model that explained methylation differences only involved geographical distance (Table S17), and not host plant (*i.e.,* consistent with an isolation-by-distance pattern, IBD; *β*GEOG*=*0.40 [0.31 – 0.49; 95% ETPI]; Fig. 5D).

Third, we found that gene ontology (GO) enrichment analyses support an element of non-randomness in the function of the DMRs. Specifically, DMRs were generally statistically enriched for functions related to protein metabolism and membrane processes, especially signal transduction and trans-membrane transport activity (Tables S18-S21).

**Discussion**

In this work, we provide an integrative approach that combines whole methylomes, genomes and transcriptomes to characterize patterns of methylation within and among natural populations of *T. cristinae.* This integrated and fine-grained strategy allowed us to detect potentially important and different patterns of methylation in nature for stick insects, which might have not been observed (*i.e.*, missed) with a less combined and coarser approach. We elaborate on this issue below.

We began by describing general patterns of methylation at species level. We reported overall genome-wide methylation level of this stick insect to be around 14%, which is considerably lower than other animals used in ecological studies (typically vertebrates, with levels ~80%) (Suzuki & Bird, 2008; Zemach *et al.*, 2010)⁠. This illustrates how having whole methylomes was likely important for having the requisite variation here to discover the patterns reported. At the same time, *T. cristinae* exhibited a level of methylation that is higher than most insects studied to date (Bewick *et al.*, 2017)⁠, but characteristic of those with incomplete metamorphosis (“Hemimetabola” insects; Provataris *et al.*, 2018)⁠. Also characteristic of this group of insects (Provataris *et al.*, 2018), methylation in *T. cristinae* mainly targeted the gene body (*i.e.* both exons and introns), and was impoverished in repeat elements (Fig. 3A). Gene body methylation is thought to stabilize and up-regulate gene expression and to reduce transcriptional noise by preventing transcription initiation outside start sites (with some suggestion it might be shaped by natural selection; (Muyle *et al.*, 2022; Neri *et al.*, 2017)⁠.

Consistent with this hypothesis, we showed methylation levels increase from 5’→3’ in gene bodies of *T. cristinae* (Fig. 3B). As longer genes are likely more prone to transcription noise, elevated methylation levels could act to suppress spurious transcription in *T. cristinae*, assuring the integrity of the genes’ function. Moreover, we found enrichment of different cellular functions depending on methylation level:genes involved in housekeeping functions were associated with increased methylation, while genes involved in signal transduction pathways showed decreased methylation levels. We detected a positive correlation between methylation and gene expression levels, which may reflect the general trend for housekeeping genes to be constitutively expressed at higher levels. However, recent studies have shown that gene body methylation has a strong dose-dependent effect on gene expression in *Arabidopsis thaliana* (He *et al.*, 2022; Shahzad *et al.*, 2021). While plants and insects are distantly related, our results suggest that gene body methylation may directly influence gene expression in other organisms, including *Timema*. Our findings therefore reveal patterns implying that methylation is associated with gene regulation in a manner that can affect higher-level phenotypes via gene expression itself that is often viewed as a ‘molecular phenotype’ (Ranz & Machado, 2006).

From our survey of methylation variation in *T. cristinae* natural populations, two different and largely contrasting patterns emerged. One is at the genome-wide scale, where overall methylation differences were observed among individuals that largely reflect their genetic relatedness and degree of geographic separation from one another in nature. Our results suggest that variation in methylation could be affected by gene flow, which decreases as geographical distance increases – following an isolation-by-distance pattern (Herrera *et al.*, 2016). In addition, such a pattern suggest a significant amount of methylation variation is due to differences in the genetic background (Adrian-Kalchhauser *et al.,* 2020; Richards, 2006; Taudt *et al.*, 2016). The relationship between methylation and genetic variation is complex. Methylation may be sequence-dependent, with certain genomic regions being more prone to methylation than others (*e.g.,* transcription factor binding sites; Onuchic et al., 2018) or be regulated by specific genic variations (*e.g.,* variants in genes encoding DNA methyltransferase; Dubin et al., 2015). On the other hand, methylation can also affect genetic variation, either directly via methylated cytosines transitioning to thymines at relatively elevated rates (*i.e.,* C-to-T mutation bias; Holliday & Grigg, 1993; Ossowski et al., 2010)⁠ or via genetic accommodation processes (Danchin et al., 2019; Klironomos et al., 2013). Therefore, there appears to be an interdependence between methylation and genetic variation, which could be at interplay in adaptive processes and which requires further studies.

The second pattern is that specific gene regions within the genome were differentially methylated between ecotypes related to the host plant that the insect uses. In contrast to genome-wide patterns, the MACAU analysis identified multiple DMRs that varied with ecotype in the wild, but not with geographic distance. Thus, different ecotypes of *T. cristinae* tend to show considerable methylation differences in DMRs regardless of whether their natal host populations are located side-by-side (*i.e.,* parapatric) or allopatric and separated by larger physical distances. Thus, these host DMRs may be viewed in some respect as methylation QTL outliers associated primarily with host plant use that were identified by ‘GWA-like’ methodology implemented in MACAU. Such a pattern could result from environmental induction and plasticity, if the methylation marks are modified directly by host-plant use. Alternatively, it could reflect divergent selection between ecotypes, if the methylation differences are transmissible between generations and affect insect fitness in a host-dependent manner. Resolving these possibilities will require future work to determine the degree to which methylation of DMRs is heritable in combination with manipulative selection experiments testing for host-associated fitness differences. It is possible that some combination of all of these processes is involved, particularly asthey are not mutually exclusive.

Our results further showed that the DMRs were widely distributed across the genome. This contrasts with the genetics of color-pattern, where loci affecting the trait are concentrated in a single region of LG8 (*Mel-Stripe*), displaying allele-frequency differences between the ecotypes (Riesch *et al.*, 2017). Therefore, it seems very likely that the association between methylation and host plants involves traits other than cryptic color-pattern and genetic regions beyond the *Mel-Stripe* locus. In this respect, our results suggested that DMRs were mostly related to protein metabolism and especially to membrane processes, including putative functions in signal transduction and ion channel activity. Among the DMRs designated by more extreme quantiles, we also detected GO terms related to synaptic processes, but the statistical power is too low to test for enrichment (*i.e.,* Tables S18-S19). Some of the genes with ion-channel activity functions could be linked to the transmission of nerve impulse (Gasque *et al.*, 2006). Indeed, DNA methylation changes in genes with these functions have been previously implicated in synaptic transmission and memory formation in mammals (Campbell & Wood, 2019; Halder *et al.*, 2015). Interestingly, there are links between differential methylation and brain plasticity in insects, related to dynamic neural circuit restructure and memory formation in honeybees (Biergans *et al.*, 2012, 2015; Maleszka, 2016). The links between these functions, differential methylation, and host-associated fitness differences is an area ripe for future study.

In conclusion, understanding the role of methylation in adaptation has been proven challenging, even in the best-studied model organisms (Heckwolf *et al.*, 2020; Hu & Barrett, 2023; Husby, 2022; Kelley *et al.*, 2021; Schmid *et al.*, 2018)⁠. Our findings therefore highlight the need for additional studies in other ecological model organisms to fully elucidate the role methylation plays in adaptation. In this regard, our current results for *T. cristinae* represent only a first step in gaining such an understanding, but an important one that lays the groundwork for exciting future research. Our identification and characterization of the pattern of DMRs within the genome and in nature positions us to target these regions to assess if the methylation differences they display are genetically based versus environmentally induced by host use, or some combination of the two. It also now sets the stage for manipulative transplant experiments in the field and lab to assess their fitness consequences and therefore effectively test for the adaptive potential of methylation changes.

**Acknowledgments.** We thank T. Oakley for lab space and H. Collin for discussions concerning epigenetics and evolution. The support and resources from the Center for High Performance Computing at the University of Utah are gratefully acknowledged, as well as access to the High Performance Computing Facilities, particularly to the Iceberg and ShARC HPC cluster, from the Corporate Information and Computing Services at the University of Sheffield. This work was funded by supporting grants from ERC NatHisGen R/129639, Royal Society of London RG140369 (C.F.d.C, P.N.), the University of Sheffield, the Human Frontier Science Program (R.R.), and FAPESP 2020/07556-8 (C.F.d.C).

**Data Accessibility.** Data, including custom code written for analyzes, have been archived in Dryad Digital Repository XXXX.

**Benefit-Sharing.** Not applicable

**Figure legends**

**Fig. 1. Factors affecting methylation variation and its consequenses for phenotypic variation.** The genetic background can influence methylation patterns. Additionally, environmental factors can affect methylation variation independently of or via an interaction with the genetic background (G x E). Knowing how methylation varies with ecological variation is another factor required to understand if and how methylation might contribute to variation in traits affecting fitness.

**Fig. 2. The *T. cristinae* study system.** (**A**) *T. cristinae* and their host plants: *Adenostoma fasciculatum* and *Ceanothus spinosus*. The ecotypes not only differ by the frequency of the dorsal white stripe, but also by differences in host-preference, body size, mate choice and cuticular hydrocarbons. (**B**) Map of the populations used in this study, selected based on host-plant species and their abundance, as well as elevation, climatic and geographic distance between populations (*Supplementary Materials*; Table S2). The general study area is situated in the Santa Ynez Moutains, in California, USA.

**Fig. 3. Comparison of methylation levels for different components of the *T. cristinae* genome.** (**A**) Methylation levels are enriched in both exons and introns (*i.e.*, the gene body) compared to genome-wide levels, and genetic repeats tend to be impoverished in methylation. (**B**) DNA methylation levels in genes and their flanking regions. The graph represents 1kbp in the 5’ downstream flanking region, multiple exons and introns in the depicted genetic region, and 1kbp in the 3’ downstream region. The graph shows mean methylation levels estimated at CpG sites found in at least 12 samples (n=14,656 genes). The x-axis represents nucleotide position from the beginning or from the end of the genomic feature. To compare exons and introns of different genes, we used the mean methylation in the first 100bp at 5’ and the last 100bp 3’ of each exon and each intron, following Glastad *et al.* (2016) and Hunt *et al.* (2013).

**Fig. 4. Genome-wide methylation differences are correlated with genetic distance.** Pairwise methylation distances were estimated using Euclidean distances between individuals, and genetic distance using the Kimura two-parameter model using GBS alignments. Regression was evaluated for significance using a Mantel test, with more complex multivariate analyses using Bayesian regression reported in the main text.

**Fig. 5. Evidence for association between methylation patterns of specific genetic regions and host plant use (i.e., differentially methylated regions, DMRs, between ecotypes).** **(A**) Manhattan plot showing association between methylation variation and ecotype across all 24 samples, for 1 kilobase-pair (kbp) tiling windows using MACAU. (Lea *et al.*, 2015). Red points represent DMRs delimited by the 0.04th quantile of the empirical *p-value* distribution (P < 0.0004, see Table S15 for details on other quantiles). Pairwise methylation distances vary mostly according to ecotype in DMRs in different quantiles of the empirical *p-value* distribution, here, represented by the (**B**) 0.04th quantile; and (**C**)0.4th quantile (also see Fig. S4, Table S16). (**D**) Genome-wide trends vary according to the geographical distances in an isolation-by-distance pattern, whereas DMRs show more of a host association. Methylation distances were obtained with Euclidean distances using the 1kbp windows in MACAU (also see Table S17).

**References**

Adrian-Kalchhauser, I., Sultan, S. E., Shama, L. N. S., Spence-Jones, H., Tiso, S., Keller Valsecchi, C. I., & Weissing, F. J. (2020). Understanding “Non-genetic” Inheritance: Insights from Molecular-Evolutionary Crosstalk. *Trends in Ecology and Evolution*, *35*(12), 1078–1089. https://doi.org/10.1016/j.tree.2020.08.011

Akalin, A., Kormaksson, M., Li, S., Garrett-Bakelman, F. E., Figueroa, M. E., Melnick, A., & Mason, C. E. (2012). MethylKit: A comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biology*, *13*(10). https://doi.org/10.1186/gb-2012-13-10-R87

Alexa, A., & Rahnenfuhrer, J. (2023). *TopGO: Enrichment Analysis for Gene Ontology* (2.52.0). doi:10.18129/B9.bioc.topGO

Anastasiadi, D., Venney, C. J., Bernatchez, L., & Wellenreuther, M. (2021). Epigenetic inheritance and reproductive mode in plants and animals. *Trends in Ecology and Evolution*, *36*(12), 1124–1140. https://doi.org/10.1016/j.tree.2021.08.006

Andrews, S., Krueger, F., Seconds-Pichon, A., Biggins, F., & Wingett, S. (2015). FastQC. A quality control tool for high throughput sequence data. Babraham Bioinformatics. In *Babraham Institute* (Vol. 1, Issue 1, p. 1). https://www.bioinformatics.babraham.ac.uk/projects/fastqc/%0Ahttp://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/

Bain, S. A., Marshall, H., de la Filia, A. G., Laetsch, D. R., Husnik, F., & Ross, L. (2021). Sex-specific expression and DNA methylation in a species with extreme sexual dimorphism and paternal genome elimination. *Molecular Ecology*, *30*(22), 5687–5703. https://doi.org/10.1111/mec.15842

Bewick, A. J., Vogel, K. J., Moore, A. J., & Schmitz, R. J. (2017). Evolution of DNA methylation across insects. *Molecular Biology and Evolution*, *34*(3), 654–665. https://doi.org/10.1093/molbev/msw264

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bossdorf, O., Richards, C. L., & Pigliucci, M. (2008). Epigenetics for ecologists. *Ecology Letters*, *11*(2), 106–115. https://doi.org/10.1111/j.1461-0248.2007.01130.x

Campbell, R. R., & Wood, M. A. (2019). How the epigenome integrates information and reshapes the synapse. *Nature Reviews Neuroscience*, *20*(3), 133–147. https://doi.org/10.1038/s41583-019-0121-9

Cedar, H., & Bergman, Y. (2009). Linking DNA methylation and histone modification: Patterns and paradigms. *Nature Reviews Genetics*, *10*(5), 295–304. https://doi.org/10.1038/nrg2540

Clarke, R. T., Rothery, P., & Raybould, A. F. (2002). Confidence limits for regression relationships between distance matrices: Estimating gene flow with distance. *Journal of Agricultural, Biological, and Environmental Statistics*, *7*(3), 361–372. https://doi.org/10.1198/108571102320

Cokus, S. J., Feng, S., Zhang, X., Chen, Z., Merriman, B., Haudenschild, C. D., Pradhan, S., Nelson, S. F., Pellegrini, M., & Jacobsen, S. E. (2008). Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. *Nature*, *452*(7184), 215–219. https://doi.org/10.1038/nature06745

Comeault, A. A., Flaxman, S. M., Riesch, R., Curran, E., Soria-Carrasco, V., Gompert, Z., Farkas, T. E., Muschick, M., Parchman, T. L., Schwander, T., Slate, J., & Nosil, P. (2015). Selection on a Genetic Polymorphism Counteracts Ecological Speciation in a Stick Insect. *Current Biology*, *25*(15), 1975–1981. https://doi.org/10.1016/j.cub.2015.05.058

Comeault, A. A., Soria-Carrasco, V., Gompert, Z., Farkas, T. E., Buerkle, C. A., Parchman, T. L., & Nosil, P. (2014). Genome-wide association mapping of phenotypic traits subject to a range of intensities of natural selection in Timema cristinae. *The American Naturalist*, *183*(5), 711–727. https://doi.org/10.1086/675497

Cubas, P., Vincent, C., & Coen, E. (1999). An epigenetic mutation responsible for natural variation in floral symmetry. *Nature*, *401*(6749), 157–161. https://doi.org/10.1038/43657

Danchin, E., Pocheville, A., Rey, O., Pujol, B., & Blanchet, S. (2019). Epigenetically facilitated mutational assimilation: Epigenetics as a hub within the inclusive evolutionary synthesis. *Biological Reviews*, *94*(1), 259–282. https://doi.org/10.1111/brv.12453

de Carvalho, C. F. (2023). Epigenetic effects of climate change on insects. *Current Opinion in Insect Science*, *57*, 101029. https://doi.org/10.1016/j.cois.2023.101029

de Mendoza, A., Hatleberg, W. L., Pang, K., Leininger, S., Bogdanovic, O., Pflueger, J., Buckberry, S., Technau, U., Hejnol, A., Adamska, M., Degnan, B. M., Degnan, S. M., & Lister, R. (2019). Convergent evolution of a vertebrate-like methylome in a marine sponge. *Nature Ecology & Evolution*, *3*(10), Article 10. https://doi.org/10.1038/s41559-019-0983-2

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

Dubin, M. J., Zhang, P., Meng, D., Remigereau, M. S., Osborne, E. J., Casale, F. P., Drewe, P., Kahles, A., Jean, G., Vilhjálmsson, B., Jagoda, J., Irez, S., Voronin, V., Song, Q., Long, Q., Rätsch, G., Stegle, O., Clark, R. M., & Nordborg, M. (2015). DNA methylation in Arabidopsis has a genetic basis and shows evidence of local adaptation. *ELife*, *4*(MAY), e05255. https://doi.org/10.7554/eLife.05255

Duncan, E. J., Gluckman, P. D., & Dearden, P. K. (2014). Epigenetics, plasticity, and evolution: How do we link epigenetic change to phenotype? *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *322*(4), 208–220. https://doi.org/10.1002/jez.b.22571

Elias, I., & Lagergren, J. (2007). Constrained hidden Markov models for population-based haplotyping. *BMC Bioinformatics*, *8*, 8–89. https://doi.org/10.1186/1471-2105-8-89

Feng, S., Cokus, S. J., Zhang, X., Chen, P.-Y., Bostick, M., Goll, M. G., Hetzel, J., Jain, J., Strauss, S. H., Halpern, M. E., Ukomadu, C., Sadler, K. C., Pradhan, S., Pellegrini, M., & Jacobsen, S. E. (2010). Conservation and divergence of methylation patterning in plants and animals. *Proceedings of the National Academy of Sciences*, *107*(19), 8689–8694. https://doi.org/10.1073/pnas.1002720107

Forester, B., Lasky, J., Wagner, H., & Urban, D. (2018). Comparing methods for detecting multilocus adaptation with multivariate genotype-environment associations. *Molecular Ecology*, *27*(9), 2215–2233. https://doi.org/10.1101/129460

Gasque, G., Labarca, P., Delgado, R., & Darszon, A. (2006). Bridging behavior and physiology: Ion-channel perspective on mushroom body-dependent olfactory learning and memory in Drosophila. *Journal of Cellular Physiology*, *209*(3), 1046–1053. https://doi.org/10.1002/jcp.20764

Glastad, K. M., Gokhale, K., Liebig, J., & Goodisman, M. A. D. (2016). The caste- and sex-specific DNA methylome of the termite Zootermopsis nevadensis. *Scientific Reports*, *6*(August), 1–14. https://doi.org/10.1038/srep37110

Glastad, K. M., Goodisman, M. A. D., Yi, S. V., & Hunt, B. G. (2016). Effects of DNA Methylation and Chromatin State on Rates of Molecular Evolution in Insects. *G3: Genes, Genomes, Genetics*, *6*(2), 357–363. https://doi.org/10.1534/g3.115.023499

Goll, M. G., & Bestor, T. H. (2005). Eukaryotic Cytosine Methyltransferases. *Annual Review of Biochemistry*, *74*(1), 481–514. https://doi.org/10.1146/annurev.biochem.74.010904.153721

Gompert, Z., Lucas, L. K., Buerkle, C. A., Forister, M. L., Fordyce, J. A., & Nice, C. C. (2014). Admixture and the organization of genetic diversity in a butterfly species complex revealed through common and rare genetic variants. *Molecular Ecology*, *23*(18), 4555–4573. https://doi.org/10.1111/mec.12811

Gompert, Z., Lucas, L. K., Nice, C. C., & Buerkle, C. A. (2013). Genome divergence and the genetic architecture of barriers to gene flow between lycaeides idas and l. Melissa. *Evolution*, *67*(9), 2498–2514. https://doi.org/10.1111/evo.12021

Gore, A. V., Tomins, K. A., Iben, J., Ma, L., Castranova, D., Davis, A. E., Parkhurst, A., Jeffery, W. R., & Weinstein, B. M. (2018). An epigenetic mechanism for cavefish eye degeneration. *Nature Ecology and Evolution*, *2*(7), 1155–1160. https://doi.org/10.1038/s41559-018-0569-4

Halder, R., Hennion, M., Vidal, R. O., Shomroni, O., Rahman, R. U., Rajput, A., Centeno, T. P., Van Bebber, F., Capece, V., Vizcaino, J. C. G., Schuetz, A. L., Burkhardt, S., Benito, E., Sala, M. N., Javan, S. B., Haass, C., Schmid, B., Fischer, A., & Bonn, S. (2015). DNA methylation changes in plasticity genes accompany the formation and maintenance of memory. *Nature Neuroscience*, *19*(1), 102–110. https://doi.org/10.1038/nn.4194

Harris, I., Jones, P. D., Osborn, T. J., & Lister, D. H. (2014). Updated high-resolution grids of monthly climatic observations—The CRU TS3.10 Dataset. *International Journal of Climatology*, *34*(3), 623–642. https://doi.org/10.1002/joc.3711

Hawes, N. A., Fidler, A. E., Tremblay, L. A., Pochon, X., Dunphy, B. J., & Smith, K. F. (2018). Understanding the role of DNA methylation in successful biological invasions: A review. *Biological Invasions*, *20*(9), 2285–2300. https://doi.org/10.1007/s10530-018-1703-6

He, L., Huang, H., Bradai, M., Zhao, C., You, Y., Ma, J., Zhao, L., Lozano-Durán, R., & Zhu, J.-K. (2022). DNA methylation-free Arabidopsis reveals crucial roles of DNA methylation in regulating gene expression and development. *Nature Communications*, *13*(1), Article 1. https://doi.org/10.1038/s41467-022-28940-2

Heckwolf, M. J., Meyer, B. S., Häsler, R., Höppner, M. P., Eizaguirre, C., & Reusch, T. B. H. (2020). Two different epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation. *Science Advances*, *6*(12). https://doi.org/10.1126/sciadv.aaz1138

Herrera, C. M., Medrano, M., & Bazaga, P. (2014). Variation in DNA methylation transmissibility, genetic heterogeneity and fecundity-related traits in natural populations of the perennial herb Helleborus foetidus. *Molecular Ecology*, *23*(5), 1085–1095. https://doi.org/10.1111/mec.12679

Herrera, C. M., Medrano, M., & Bazaga, P. (2016). Comparative spatial genetics and epigenetics of plant populations: Heuristic value and a proof of concept. *Molecular Ecology*, *25*(8), 1653–1664. https://doi.org/10.1111/mec.13576

Holliday, R., & Grigg, G. W. (1993). DNA methylation and mutation. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *285*(1), 61–67. https://doi.org/10.1016/0027-5107(93)90052-H

Hu, J., & Barrett, R. D. H. (2017). Epigenetics in natural animal populations. *Journal of Evolutionary Biology*, *30*(9), 1612–1632. https://doi.org/10.1111/jeb.13130

Hu, J., & Barrett, R. D. H. (2023). The role of plastic and evolved DNA methylation in parallel adaptation of threespine stickleback (Gasterosteus aculeatus). *Molecular Ecology*, *32*(7), 1581–1591. https://doi.org/10.1111/mec.16832

Hu, J., Pérez-Jvostov, F., Blondel, L., & Barrett, R. D. H. (2018). Genome-wide DNA methylation signatures of infection status in Trinidadian guppies ( *Poecilia reticulata* ). *Molecular Ecology*, *27*(15), 3087–3102. https://doi.org/10.1111/mec.14771

Hu, J., Wuitchik, S. J. S., Barry, T. N., Jamniczky, H. A., Rogers, S. M., & Barrett, R. D. H. (2021). Heritability of DNA methylation in threespine stickleback ( Gasterosteus aculeatus ). *Genetics*, *217*(1). https://doi.org/10.1093/GENETICS/IYAB001

Hunt, B. G., Glastad, K. M., Yi, S. V., & Goodisman, M. A. D. (2013). Patterning and regulatory associations of DNA methylation are mirrored by histone modifications in insects. *Genome Biology and Evolution*, *5*(3), 591–598. https://doi.org/10.1093/gbe/evt030

Husby, A. (2022). Wild epigenetics: Insights from epigenetic studies on natural populations. *Proceedings of the Royal Society B: Biological Sciences*, *289*(1968). https://doi.org/10.1098/rspb.2021.1633

Kelley, J. L., Tobler, M., Beck, D., Sadler-Riggleman, I., Quackenbush, C. R., Arias Rodriguez, L., & Skinner, M. K. (2021). Epigenetic inheritance of DNA methylation changes in fish living in hydrogen sulfide–rich springs. *Proceedings of the National Academy of Sciences*, *118*(26), e2014929118. https://doi.org/10.1073/pnas.2014929118

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*(2), 111–120. https://doi.org/10.1007/BF01731581

Klironomos, F. D., Berg, J., & Collins, S. (2013). How epigenetic mutations can affect genetic evolution: Model and mechanism. *BioEssays*, *35*(6), 571–578. https://doi.org/10.1002/bies.201200169

Krueger, F., & Andrews, S. R. (2011). Bismark: A flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*, *27*(11), 1571–1572. https://doi.org/10.1093/bioinformatics/btr167

Law, J. A., & Jacobsen, S. E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nature Reviews Genetics*, *11*(3), 204–220. https://doi.org/10.1038/nrg2719

Lea, A. J., Altmann, J., Alberts, S. C., & Tung, J. (2016). Resource base influences genome-wide DNA methylation levels in wild baboons (Papio cynocephalus). *Molecular Ecology*, *25*(8), 1681–1696. https://doi.org/10.1111/mec.13436

Lea, A. J., Tung, J., & Zhou, X. (2015). A Flexible, Efficient Binomial Mixed Model for Identifying Differential DNA Methylation in Bisulfite Sequencing Data. *PLoS Genetics*, *11*(11), 1–31. https://doi.org/10.1371/journal.pgen.1005650

Lea, A. J., Vilgalys, T. P., Durst, P. A. P., & Tung, J. (2017). Maximizing ecological and evolutionary insight in bisulfite sequencing data sets. *Nature Ecology & Evolution*, *1*(8), 1074–1083. https://doi.org/10.1038/s41559-017-0229-0

Ledón-Rettig, C. C. (2013). Ecological epigenetics: An introduction to the symposium. *Integrative and Comparative Biology*, *53*(2), 307–318. https://doi.org/10.1093/icb/ict053

Lewis, S. H., Ross, L., Bain, S. A., Pahita, E., Smith, S. A., Cordaux, R., Miska, E. A., Lenhard, B., Jiggins, F. M., & Sarkies, P. (2020). Widespread conservation and lineage-specific diversification of genome-wide DNA methylation patterns across arthropods. *PLoS Genetics*, *16*(6). https://doi.org/10.1371/journal.pgen.1008864

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., & Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Liao, Y., Smyth, G. K., & Shi, W. (2014). FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, *30*(7), 923–930. https://doi.org/10.1093/bioinformatics/btt656

Lindner, M., Verhagen, I., Viitaniemi, H. M., Laine, V. N., Visser, M. E., Husby, A., & van Oers, K. (2021). Temporal changes in DNA methylation and RNA expression in a small song bird: Within- and between-tissue comparisons. *BMC Genomics*, *22*(1), 36. https://doi.org/10.1186/s12864-020-07329-9

Lindtke, D., Lucek, K., Soria-Carrasco, V., Villoutreix, R., Farkas, T. E., Riesch, R., Dennis, S. R., Gompert, Z., & Nosil, P. (2017). Long-term balancing selection on chromosomal variants associated with crypsis in a stick insect. *Molecular Ecology*, *26*(22), 6189–6205. https://doi.org/10.1111/mec.14280

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 1–21. https://doi.org/10.1186/s13059-014-0550-8

McGaughey, D. M., Abaan, H. O., Miller, R. M., Kropp, P. A., & Brody, L. C. (2014). Genomics of CpG Methylation in Developing and Developed Zebrafish. *G3 Genes|Genomes|Genetics*, *4*(5), 861–869. https://doi.org/10.1534/g3.113.009514

McGuigan, K., Hoffmann, A. A., & Sgrò, C. M. (2021). How is epigenetics predicted to contribute to climate change adaptation? What evidence do we need? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *376*(1826). https://doi.org/10.1098/rstb.2020.0119

Metzger, D. C. H., & Schulte, P. M. (2018). Similarities in temperature-dependent gene expression plasticity across timescales in threespine stickleback (Gasterosteus aculeatus). *Molecular Ecology*, *27*(10), 2381–2396. https://doi.org/10.1111/mec.14591

Morgan, H. D., Sutherland, H. G. E., Whitelaw, E., & Martin, D. I. K. (1999). Epigenetic inheritance at the agouti locus in the mouse. *Nature Genetics*, *23*(3), 314–318. https://doi.org/10.1038/15490

Muyle, A. M., Seymour, D. K., Lv, Y., Huettel, B., & Gaut, B. S. (2022). Gene Body Methylation in Plants: Mechanisms, Functions, and Important Implications for Understanding Evolutionary Processes. *Genome Biology and Evolution*, *14*(4), 1–18. https://doi.org/10.1093/gbe/evac038

Neri, F., Rapelli, S., Krepelova, A., Incarnato, D., Parlato, C., Basile, G., Maldotti, M., Anselmi, F., & Oliviero, S. (2017). Intragenic DNA methylation prevents spurious transcription initiation. *Nature*, *543*(7643), Article 7643. https://doi.org/10.1038/nature21373

Nosil, P. (2007). Divergent host plant adaptation and reproductive isolation between ecotypes of Timema cristinae walking sticks. *The American Naturalist*, *169*(2), 151–162. https://doi.org/10.1086/510634

Nosil, P., & Crespi, B. J. (2006). Experimental evidence that predation promotes divergence in adaptive radiation. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(24), 9090–9095. https://doi.org/10.1073/pnas.0601575103

Nosil, P., Crespi, B. J., & Sandoval, C. P. (2002). Host-plant adaptation drives the parallel evolution of reproductive isolation. *Nature*, *417*(6887), 440–443. https://doi.org/10.1038/417440a

Nosil, P., Gompert, Z., Farkas, T. E., Comeault, A. A., Feder, J. L., Buerkle, C. a., & Parchman, T. L. (2012). Genomic consequences of multiple speciation processes in a stick insect. *Proceedings of the Royal Society B: Biological Sciences*, *June*, 5058–5065. https://doi.org/10.1098/rspb.2012.0813

Nosil, P., Sandoval, C. P., & Crespi, B. J. (2006). The evolution of host preference in allopatric vs. Parapatric populations of Timema cristinae walking-sticks. *Journal of Evolutionary Biology*, *19*(3), 929–942. https://doi.org/10.1111/j.1420-9101.2005.01035.x

Nosil, P., Villoutreix, R., de Carvalho, C. F., Farkas, T. E., Soria-Carrasco, V., Feder, J. L., Crespi, B. J., & Gompert, Z. (2018). Natural selection and the predictability of evolution in Timema stick insects. *Science (New York, N.Y.)*, *359*(6377), 765–770. https://doi.org/10.1126/science.aap9125

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., & et al. (2022). *The vegan package*. https://cran.r-project.org/package=vegan

Onuchic, V., Lurie, E., Carrero, I., Pawliczek, P., Patel, R. Y., Rozowsky, J., Galeev, T., Huang, Z., Altshuler, R. C., Zhang, Z., Harris, R. A., Coarfa, C., Ashmore, L., Bertol, J. W., Fakhouri, W. D., Yu, F., Kellis, M., Gerstein, M., & Milosavljevic, A. (2018). Allele-specific epigenome maps reveal sequence-dependent stochastic switching at regulatory loci. *Science*, *1354*(September), eaar3146. https://doi.org/10.1126/science.aar3146

Ossowski, S., Schneeberger, K., Lucas-Lledó, J. I., Warthmann, N., Clark, R. M., Shaw, R. G., Weigel, D., & Lynch, M. (2010). The Rate and Molecular Spectrum in Arabidopsis thaliana. *Science*, *327*(January), 92–95.

Parchman, T. L., Gompert, Z., Mudge, J., Schilkey, F. D., Benkman, C. W., & Buerkle, C. A. (2012). Genome-wide association genetics of an adaptive trait in lodgepole pine. *Molecular Ecology*, *21*(12), 2991–3005. https://doi.org/10.1111/j.1365-294X.2012.05513.x

Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S., & Hoekstra, H. E. (2012). Double digest RADseq: An inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS ONE*, *7*(5). https://doi.org/10.1371/journal.pone.0037135

Plummer, Martyn. (2018). rjags: Bayesian Graphical Models using MCMC. *R Package Version 4-8.* https://cran.r-project.org/package=rjags

Provataris, P., Meusemann, K., Niehuis, O., Grath, S., & Misof, B. (2018). Signatures of DNA methylation across insects suggest reduced DNA methylation levels in Holometabola. *Genome Biology and Evolution*, *10*(March), 1185–1197. https://doi.org/10.1093/gbe/evy066/4943971

R Core Team. (2020). *R: A language and environment for statistical computing.* https://www.r-project.org/

Ranz, J. M., & Machado, C. A. (2006). Uncovering evolutionary patterns of gene expression using microarrays. *Trends in Ecology & Evolution*, *21*(1), 29–37. https://doi.org/10.1016/j.tree.2005.09.002

Richards, C. L., Alonso, C., Becker, C., Bossdorf, O., Bucher, E., Colomé-Tatché, M., Durka, W., Engelhardt, J., Gaspar, B., Gogol-Döring, A., Grosse, I., van Gurp, T. P., Heer, K., Kronholm, I., Lampei, C., Latzel, V., Mirouze, M., Opgenoorth, L., Paun, O., … Verhoeven, K. J. F. (2017). Ecological plant epigenetics: Evidence from model and non-model species, and the way forward. *Ecology Letters*, *20*(12), 1576–1590. https://doi.org/10.1111/ele.12858

Richards, E. J. (2006). Inherited epigenetic variation—Revisiting soft inheritance. *Nature Reviews Genetics*, *7*(5), 395–401. https://doi.org/10.1038/nrg1834

Riesch, R., Muschick, M., Lindtke, D., Villoutreix, R., Comeault, A. A., Farkas, T. E., Lucek, K., Hellen, E., Soria-Carrasco, V., Dennis, S. R., De Carvalho, C. F., Safran, R. J., Sandoval, C. P., Feder, J., Gries, R., Crespi, B. J., Gries, G., Gompert, Z., & Nosil, P. (2017). Transitions between phases of genomic differentiation during stick-insect speciation. *Nature Ecology and Evolution*, *1*(4), 1–13. https://doi.org/10.1038/s41559-017-0082

Rousset, F. (1997). Genetic Differentiation and estimation of Gene Flow from F-Statistics Under Isolation by Distance. *Genetics*, *145*, 1219–1228. https://doi.org/10.1007/BF00329997

Sagonas, K., Meyer, B. S., Kaufmann, J., Lenz, T. L., Häsler, R., & Eizaguirre, C. (2020). Experimental Parasite Infection Causes Genome-Wide Changes in DNA Methylation. *Molecular Biology and Evolution*, *37*(8), 2287–2299. https://doi.org/10.1093/molbev/msaa084

Sandoval, C. P. (1994a). Differential visual predation on morphs of Timema cristinae (Phasmatodeae:Timemidae) and its consequences for host range. *Biological Journal of the Linnean Society*, *52*, 341–356.

Sandoval, C. P. (1994b). The effects of the relative geographic scales of gene flow and selection on morph frequencies in the walking-stick Timema cristinae. *Evolution*, *48*(6), 1866–1879. https://doi.org/10.1111/j.1558-5646.1994.tb02220.x

Schmid, M. W., Heichinger, C., Coman Schmid, D., Guthörl, D., Gagliardini, V., Bruggmann, R., Aluri, S., Aquino, C., Schmid, B., Turnbull, L. A., & Grossniklaus, U. (2018). Contribution of epigenetic variation to adaptation in Arabidopsis. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-06932-5

Shahzad, Z., Moore, J. D., Choi, J., & Zilberman, D. (2021). *Epigenetic inheritance mediates phenotypic diversity in natural populations* (p. 2021.03.15.435374). bioRxiv. https://doi.org/10.1101/2021.03.15.435374

Simonsen, M., Mailund, T., & Pedersen, C. N. S. (2008). Rapid Neighbour-Joining. *Proceedings of the 8th Workshop in Algorithms in Bioinformatics*, 113–122. https://doi.org/doi:10.1007/978-3-540-87361-7\_10

Simonsen, M., & Pedersen, C. N. S. (2011). Rapid computation of distance estimators from nucleotide and amino acid alignments. *Proceedings of the ACM Symposium on Applied Computing*, *1*, 89–93. https://doi.org/10.1145/1982185.1982208

Soria-Carrasco, V., Gompert, Z., Comeault, A. a., Farkas, T. E., Parchman, T. L., Johnston, J. S., Buerkle, C. A., Feder, J. L., Bast, J., Schwander, T., Egan, S. P., Crespi, B. J., & Nosil, P. (2014). Stick insect genomes reveal natural selection’s role in parallel speciation. *Science (New York, N.Y.)*, *344*(6185), 738–742. https://doi.org/10.1126/science.1252136

Stajic, D., & Jansen, L. E. T. (2021). Empirical evidence for epigenetic inheritance driving evolutionary adaptation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *376*(1826). https://doi.org/10.1098/rstb.2020.0121

Suzuki, M. M., & Bird, A. (2008). DNA methylation landscapes: Provocative insights from epigenomics. *Nature Reviews Genetics*, *9*(6), 465–476. https://doi.org/10.1038/nrg2341

Taudt, A., Colomé-Tatché, M., & Johannes, F. (2016). Genetic sources of population epigenomic variation. *Nature Reviews Genetics*, *17*(6), 319–332. https://doi.org/10.1038/nrg.2016.45

van der Graaf, A., Wardenaar, R., Neumann, D. A., Taudt, A., Shaw, R. G., Jansen, R. C., Schmitz, R. J., Colomé-Tatché, M., & Johannes, F. (2015). Rate, spectrum, and evolutionary dynamics of spontaneous epimutations. *Proceedings of the National Academy of Sciences*, *112*(21), 6676–6681. https://doi.org/10.1073/pnas.1424254112

Verhoeven, K. J. F., VonHoldt, B. M., & Sork, V. L. (2016). Epigenetics in ecology and evolution: What we know and what we need to know. *Molecular Ecology*, *25*(8), 1631–1638. https://doi.org/10.1111/mec.13617

Vickery, V. R. (1993). Vickery, V. R. (1993). Revision of Timema Scudder (Phasmatoptera: Timematodea) including three new species. *The Canadian Entomolgist*, *125*(4), 657–692.

Villoutreix, R., de Carvalho, C. F., Soria-Carrasco, V., Lindtke, D., De-la-Mora, M., Muschick, M., Feder, J. L., Parchman, T. L., Gompert, Z., & Nosil, P. (2020). Large-scale mutation in the evolution of a gene complex for cryptic coloration. *Science*, *369*, 460–466.

Yagound, B., Remnant, E. J., Buchmann, G., & Oldroyd, B. P. (2020). Intergenerational transfer of DNA methylation marks in the honey bee. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(51), 32519–32527. https://doi.org/10.1073/pnas.2017094117

Yao, N., Schmitz, R. J., & Johannes, F. (2021). Epimutations Define a Fast-Ticking Molecular Clock in Plants. *Trends in Genetics*, *37*(8), 699–710. https://doi.org/10.1016/j.tig.2021.04.010

Yu, X., Marshall, H., Liu, Y., Xiong, Y., Zeng, X., Yu, H., Chen, W., Zhou, G., Zhu, B., Ross, L., & Lu, Z. (2023). Sex-specific transcription and DNA methylation landscapes of the Asian citrus psyllid, a vector of huanglongbing pathogens. *Evolution*, *77*(5), 1203–1215. https://doi.org/10.1093/evolut/qpad036

Zemach, A., McDaniel, I. E., Silva, P., & Zilberman, D. (2010). Genome-wide evolutionary analysis of eukaryotic DNA methylation. *Science (New York, N.Y.)*, *328*(5980), 916–919. https://doi.org/10.1126/science.1186366

Zhang, H., Lang, Z., & Zhu, J. K. (2018). Dynamics and function of DNA methylation in plants. *Nature Reviews Molecular Cell Biology*, *19*(8), 489–506. https://doi.org/10.1038/s41580-018-0016-z

Zhou, X., Carbonetto, P., & Stephens, M. (2013). Polygenic Modeling with Bayesian Sparse Linear Mixed Models. *PLoS Genetics*, *9*(2), e1003264. https://doi.org/10.1371/journal.pgen.1003264