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Author for correspondence:
Clarissa F. de Carvalho
e-mail: clarissa.decarvalho@gwu.edu

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Genomic regions exhibiting divergent DNA methylation patterns co-vary with loci associated with sexual signalling traits in a stick insect

Clarissa F. de Carvalho[1,2], Nicholas P. Planidin[3], Jon Slate[1], Rüdiger Riesch[4], Zach Gompert[5] and Patrik Nosil[1,3]

[1]School of Biosciences, The University of Sheffield, Sheffield, England, UK
[2]Biological Sciences Department, The George Washington University, WA, DC, USA
[3]Theoretical and Experimental Ecology Station, Moulis, Occitanie, France
[4]Department of Biological Sciences, Centre for Ecology, Evolution and Behaviour, Royal Holloway University of London, Egham, England, UK
[5]Department of Biological Sciences, Utah State University, Logan, UT, USA

CFdC, 0000-0002-5780-5454; NPP, 0000-0003-4182-0841; JS, 0000-0003-3356-5123; RR, 0000-0002-0223-1254; PN, 0000-0002-8271-9005

The evolution of signalling traits can affect local adaptation, mate choice and the development of reproductive isolation between diverging populations. While the genetic basis of many sexual signalling traits is well characterized, variation in these traits may also reflect regulatory mechanisms, including epigenetic marks. However, the extent to which epigenetic marks, particularly DNA methylation, are associated with signalling traits remains unclear. Here, we focus on epigenetic variation and cuticular hydrocarbons (CHCs), the latter being chemical traits that can be used for mate choice in insects. Specifically, we investigate the association between DNA methylation and loci associated with CHC variation in *Timema cristinae* stick insects. We integrate previously published analyses of differentially methylated regions (DMRs) between host-plant ecotypes with genome-wide association results for CHCs. We find that DMRs co-vary with genetic loci associated with pentacosanes, the CHC class most strongly implicated in mate choice and sexual isolation in this system. Our documented association between DNA methylation and signalling traits suggests a possible role for epigenetic marks in mate choice and population divergence. Future studies that establish causal links between DNA methylation and signal trait variation are required to clarify the contribution of methylation to mate choice, prezygotic isolation, and ultimately, speciation.

This article is part of the theme issue 'Ecological epigenetics at the intersection of behaviour and life history variation in non-model animals'.

1. Introduction

The origin of new species involves the development of barriers that create reproductive isolation between populations [1–3]. Such barriers often emerge through divergence in traits that mediate behavioural interactions between individuals, including signals such as colouration, pheromones, songs and acoustic displays [4–9]. Given that variation in signalling traits can influence mate choice, their divergence is often shaped by sexual selection and can reduce gene flow, contributing to sexual isolation [10]. As a result, understanding sexual

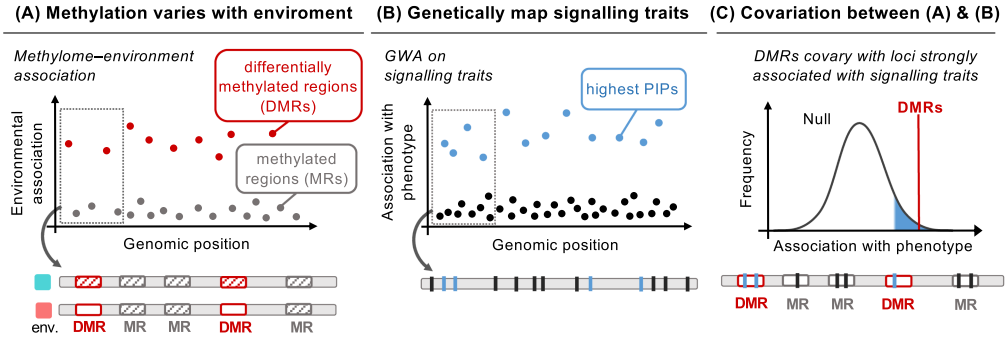

Figure 1. Strategy to test whether environmentally associated DNA methylation patterns co-vary with genetic regions associated with signalling traits. (A) Identify genomic regions for which DNA methylation varies with environmental gradients (i.e. differentially methylated regions, DMRs), for example, using methylome–environment association studies. The schematic at the bottom illustrates methylation patterns across populations inhabiting two different environments (env.), highlighting regions with similar methylation patterns (methylated regions, MRs) and regions showing environmentally associated differences in methylation (DMRs). (B) Map of genetic regions associated with signalling traits using genome-wide association studies (GWA). The strength of the association can be measured using statistics such as posterior inclusion probabilities (PIPs) from Bayesian sparse linear mixed models. The schematic at the bottom illustrates the genomic distribution of loci with varying PIP values, with blue indicating stronger associations. (C) Test for no-random co-variation between DMRs and loci most strongly associated with signal traits. This can be done by evaluating whether DMRs show: (i) higher summed PIPs, (ii) higher mean PIPs and (iii) more single-nucleotide polymorphisms (SNPs) in the top 5% of PIPs than expected by chance. The schematic at the bottom illustrates DMRs tending to coincide with SNPs with the strongest associations to signalling traits.

signalling traits (signalling traits, hereafter) can offer key insights into the mechanisms by which reproductive barriers arise during population divergence [11–14].

Signalling traits themselves can be shaped by their genetic architecture [15,16]. Accordingly, numerous studies have examined their genetic basis within and among different taxa, including traits such as colouration [17–22], acoustic signals [23,24], morphological features [25,26] and chemical cues [27,28]. In addition, signalling traits and mate preferences can be sensitive to environmental and physiological conditions. This can result in context- and condition-dependent variation in signalling traits across space and time [16,29–31]. For example, such plasticity may reflect the individual's condition [32–35], as illustrated by condition-dependent traits such as carotenoid-based colouration [36–38]. More generally, plasticity in signalling traits can arise through multiple mechanisms, including changes in the regulation of gene expression in response to environmental cues.

In this context, epigenetic marks may contribute to variation in signalling traits by acting as a regulatory layer through which genetic and environmental information are integrated [39]. Because epigenetic marks can mediate genotype–environment interactions [40,41], they represent a potential molecular mechanism contributing to phenotypic plasticity of signalling traits [42]. However, whether epigenetic variation is associated with signalling traits remains poorly understood. Addressing this gap may help clarify how genetic and environmental factors can jointly contribute to variation in sexually relevant signals during population divergence and reproductive isolation, motivating the present study.

This study focuses on DNA methylation, the most extensively studied epigenetic mark in ecological and evolutionary research [43,44]. DNA methylation involves the addition of a methyl group to a cytosine nucleotide [45], which can affect gene expression by altering chromatin accessibility and transcriptional activity [46,47]. Importantly, DNA methylation can persist through mitotic divisions and, in some cases, through meiotic divisions—potentially transmitting its phenotypic effects to the next generation (e.g. [48–51]). Patterns of DNA methylation can be shaped by both genetic background and environmental conditions [52–55] and may contribute to phenotypic plasticity [41,56,57]. In this way, variation in DNA methylation and its phenotypic effects can arise from genetic effects, environmental effects or genotype-by-environment interactions (G × E) [40,53]. While several studies have examined links between DNA methylation and sexual dimorphism (e.g. [58–60]), its association with variation in signalling traits remains unclear.

Here, we begin to address this gap by testing whether genomic regions exhibiting environmentally divergent DNA methylation patterns are associated with genetic variation underlying signalling traits. We did so using a three-step framework. First, we assessed DNA methylation patterns among populations to identify regions where methylation varies with environmental conditions (i.e. potentially reflecting associations with locally adapted genetic variants, environmental influences or genotype-by-environment interactions; figure 1A). Second, we used results from genetic mapping of signalling traits to identify loci whose variation is most strongly associated with trait variation. While this approach targets the heritable component of phenotypic variation, the identified regions may also contribute to phenotypic plasticity (e.g. through regulation by DNA methylation, figure 1B). Third, we tested whether genomic regions exhibiting environmentally associated DNA methylation patterns co-vary, more than expected by chance, with loci associated with signalling traits (figure 1C). This approach highlights candidate regions where epigenetic marks are associated with signalling traits, rather than evaluating direct causal effects of epigenetic marks on these traits.

We apply this framework in *Timema cristinae* stick insects [61]. This species is primarily found on two host-plant species: *Adenostoma fasciculatum* (Rosaceae) and *Ceanothus spinosus* (Rhamnaceae) in chaparral habitats of the Santa Ynez Mountains in California, USA (figure 2A) [62,63]. Host-associated divergent selection contributes to partial reproductive isolation in this species,

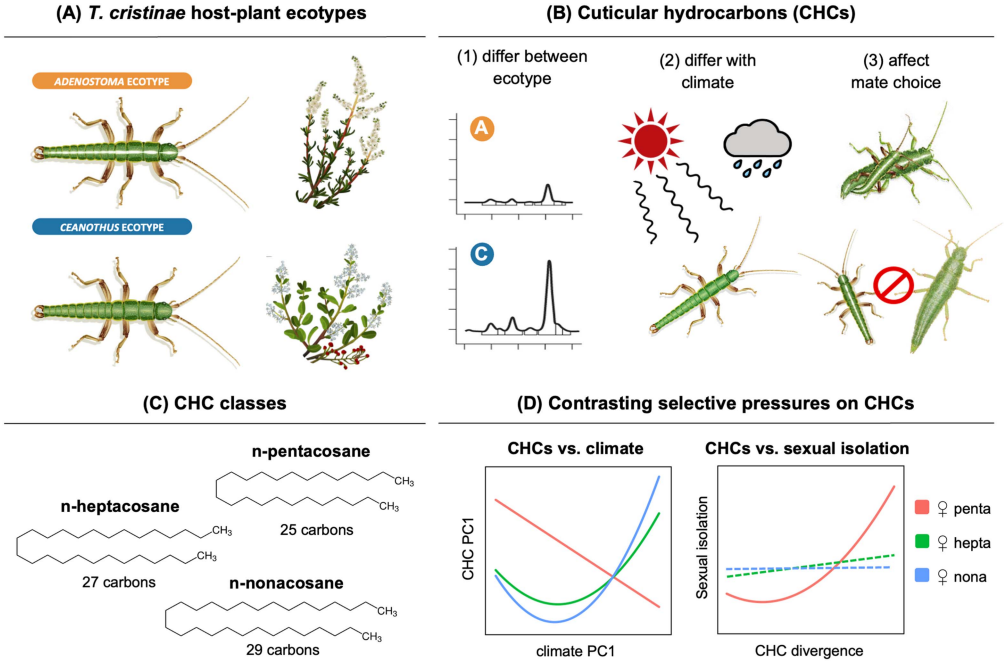

Figure 2. The *T. cristinae* study system. (A) *Timema cristinae* and their host plants: *A. fasciculatum* and *C. spinosus*. The ecotypes are distinguished by several traits, including colour-pattern, body size, host preference and cuticular hydrocarbon profiles. (B) Properties of cuticular hydrocarbons (CHCs) in *T. cristinae*. CHCs play a key role in desiccation resistance and mate recognition in insects. CHC profiles vary with (1) host-plant ecotype and (2) across climatic conditions; and (3) are used in mate choice and contribute to sexual isolation among *Timema* populations and species. (C) Three classes of CHC compounds have been studied in *T. cristinae*, classified by their chain length: penta-, hepta- and nonacosanes (25, 27 and 29 carbon chains, respectively [71–73]). (D) While all CHC classes correlate with climatic variation, divergence in pentacosanes is strongly linked to sexual isolation, whereas hepta- and nonacosanes show no such association (represented by dashed lines) [73]. The graphs shown were modified from the results of [73]. Abbreviations: penta = pentacosanes, hepta = heptacosanes and nona = nonacosanes.

giving rise to *Adenostoma* and *Ceanothus* ecotypes [64]. A component of such reproductive isolation is sexual isolation between ecotypes, in which males prefer females from their same ecotype, and prefer conspecific females over heterospecifics [65,66].

Key traits underlying both adaptation and sexual isolation in *T. cristinae* are the cuticular hydrocarbons (CHCs)—chemical traits that are involved, among other things, in desiccation resistance and mate recognition in insects (figure 2B) [67]. CHCs are partially plastic, varying with environmental factors like diet and climate in several insect species [68,69]. In *T. cristinae*, these compounds differ between host-plant ecotypes and are associated with climatic variation [70–72]. CHCs are also known to be under polygenic control and moderately heritable in this system [71,72]. However, the molecular and regulatory mechanisms through which environmental variation contributes to CHC variation, and how these effects interact with the genetic architecture, remain poorly understood. As a result, environmentally associated CHC variation may arise through multiple, non-mutually exclusive processes, including genetic divergence, environmentally induced plasticity and regulatory mechanisms that mediate environmental effects on gene expression.

Female CHC profiles also function as sexual signalling traits that influence male mate choice and contribute to sexual isolation both within and between *Timema* species [70]. For example, greater divergence in female CHC profiles is associated with stronger sexual isolation at both intra- and interspecific levels [70,71]. In addition, a causal role for CHCs in mate choice has been demonstrated through manipulative perfuming experiments, in which experimentally altering CHC profiles affected mate choice within and between species [71]. Most critically, the strength of association with mate choice and sexual isolation differs among CHC classes. Specifically, shorter-chain compounds (pentacosanes) show the strongest association with sexual isolation among populations, whereas longer-chain compounds (heptacosanes and nonacosanes) are more closely linked to climatic variation and desiccation resistance (figure 2C,D) [73].

Given their polygenic architecture, role in sexual signalling and likely sensitivity to environmental variation, CHCs provide a suitable system for testing associations with environmentally associated regulatory variation, specifically DNA methylation patterns. As such, using the framework outlined above (figure 1), we evaluated whether genomic regions that show differential methylation between ecotypes co-vary with loci strongly associated with CHC variation more than expected by chance. In particular, given the stronger empirical association between pentacosanes and sexual isolation in this system (figure 2D) [71,73], we here treated pentacosanes as the focal signalling trait, while using heptacosanes and nonacosanes as contrasts to assess the specificity of any detected co-variation. To this end, we leveraged previously published results on genome-wide association (GWA) and methylome–environment association that identified CHC-associated loci and ecotype-associated DNA methylation patterns, respectively [71,72,74], to test for co-variation between these genomic features. The goal of this study was to take a first step towards understanding the relationship between environmentally associated DNA methylation patterns and loci associated with sexual signalling traits by testing for non-random patterns rather than inferring causality. By focusing on CHCs as both sexually

selected and ecologically relevant traits, our study provides initial insights into how epigenetic marks and genetic variation may jointly explain patterns of signalling relevant to mate choice and reproductive isolation.

2. Material and methods

(a) Ecotype-associated differentially methylated regions in *Timema cristinae*

We began our investigation by focusing on DNA methylation patterns associated with host-plant ecotype. To this end, we used previously published results from [74]. This study assessed DNA methylation differences between host-plant ecotypes of *T. cristinae* based on whole-genome bisulfite sequencing (WGBS) data from 24 female adult individuals (two per population), sampled from 12 populations in the Spring of 2017 (supplementary material, figure S1). The sampled populations are characterized by their different abundances of *Adenostoma* and *Ceanothus*. Reads were mapped to the *T. cristinae* reference genome v1.3c2 [75], and CpG sites overlapping known single-nucleotide polymorphisms (SNPs) were excluded to minimize confounding effects on methylation calls [74].

Ecotype-associated differentially methylated regions (DMRs) were identified in [74] using methylome–environment association analyses (MACAU v.1.0.0; [76]), applied to non-overlapping 1 kilobasepair (kbp) methylation tilling windows. Rather than defining one single significance threshold, the authors designated DMRs based on different percentiles of the tail of the empirical *p*-value distribution, specifically the 0.04th to the 0.40th percentile of the distribution ($p = 0.0004$ to 0.0061). These empirical *p*-value cut-offs yielded between 25 and 258 DMRs, depending on the stringency.

We here highlight some properties of *T. cristinae*'s DNA methylation biology and ecotype-associated DMRs that are relevant to the present study [74]. First, there is a general positive association between gene-body DNA methylation levels and gene expression, suggesting that variation in genic methylation may be functionally relevant. Second, DMRs were identified based on their association with host-plant ecotype and thus show stronger associations with host-plant ecotype than with genetic, geographic or climatic variation across the different *p*-value cut-offs. In addition, DMRs were distributed across multiple chromosomes and overlapped at annotated genes at a rate of approximately 72%. This is comparable to the proportion of 1 kbp tiles used for methylome–environment analyses that overlapped with genes (approx. 74%). We used this DMR dataset to estimate the co-variation with genomic regions associated with pentacosanes.

(b) Genomic mapping of variation in cuticular hydrocarbon traits

To identify genomic regions associated with variation in pentacosanes, as well as in hepta- and nonacosanes, we used previously published results on GWA mapping for *T. cristinae* [72]. In that study, the authors themselves reanalysed previously published genetic and phenotypic data from [71] with updated genomic resources. Details of the original GWA analyses are provided below for completeness.

The CHC phenotypic and genotypic data used in these studies were obtained from wild individuals in the Spring of 2013 (FH, *Adenostoma*, latitude 34.518°, longitude –119.801°, included in the WGBS sequencing, supplementary material, figure S1) [71]. This population has a large population size (with an estimated effective population size of approximately 110 and the census size being one order of magnitude larger) and shows no evidence of internal genetic structure [71,73,77,78]. Their phenotyping yielded relative proportions of the three CHC classes that differ in chain length: pentacosanes, heptacosanes and nonacosanes (i.e. chains of 25, 27 and 29 hydrocarbons, respectively; see supplementary material for details on CHC phenotyping). Genetic sequencing was done by Riesch *et al*. [71] using genotype-by-sequencing for these same phenotyped individuals (175, 918, SNPs) and Chaturvedi *et al*. [72] performed mapping to *T. cristinae* reference genome v1.3c2, variant calling and genotyping.

In this work, we focused exclusively on the results for female adult individuals ($n = 197$), as female CHC profiles have been shown to mediate male mate choice and contribute to sexual reproductive isolation [71,72]. We used the GWA results from [72] for each CHC class, obtained through Bayesian sparse linear mixed models implemented in the software *GEMMA* [79]. These models estimate the proportion of variation explained by the genetic data for each trait (which should approximate the trait's heritability) and assign posterior inclusion probabilities (PIP) to individual SNPs. The PIPs represent the probability that a given SNP is associated with phenotypic variation. Briefly, the analyses were conducted on 10 independent Markov chain Monte Carlo chains per trait (1 000 000 sampling steps; 200 000 burn-in; minor allele frequency threshold = 0) [72]. Median proportions of phenotypic variance explained by all SNPs were 89.7% for pentacosanes (95% equal-tail probability interval (ETPI) 35.8–99.9), 52.5% for heptacosanes (95% ETPI: 4.9–98.9) and 80.2% for nonacosanes (95% ETPI: 15.5–99.8) (supplementary material, table S1).

(c) Identifying associations between host-ecotype differentially methylated regions, cuticular hydrocarbons and single-nucleotide polymorphisms

We here tested for co-variation between DMRs and genomic regions associated with variation in each CHC class using the two datasets described above. We predicted that DMRs would show higher overall levels of association with pentacosane-associated loci than expected by chance, whereas we expected weaker or no associations for other CHC classes, which are used for comparison [73]. To test this prediction, we used the PIP estimates from the GWA analyses to quantify the degree of association

between phenotypic variation in each CHC class and SNPs [72]. Associations were evaluated only for SNPs located within the 1 kbp methylation windows used in the methylome–environment analyses [74].

Here, DMRs were designated using four *p*-value cut-offs from the empirical distribution of the methylome–environment association analyses [74]. We adopted the most stringent (0.04th percentile, $p < 0.0004$), an intermediate (0.20^{th} percentile, $p < 0.0028$) and the more relaxed (0.40^{th} percentile, $p < 0.0061$) thresholds among the *p*-value cut-offs used in [74]. We further considered the nominal significance level of $p < 0.01$ as the upper bound of interest. This range of *p*-value cut-offs allowed us to assess the robustness of DMR–CHC associations, serving as a sensitivity analysis.

We assessed the degree of association between CHC-associated loci and DMRs using two complementary metrics. First, we calculated the sum of PIP values for all SNPs located within DMRs. This metric captures the total strength of genetic association accumulated within DMRs. Second, we calculated the mean PIP value of SNPs located within DMRs, capturing the average strength of association within these regions. Elevated values in either metric relative to null expectations would indicate a non-random association between DMRs and CHC-associated loci. Although the CHC and DNA methylation datasets differ in sampling design, their comparison is appropriate for a pattern-based test of co-variation. Moreover, this approach is probably conservative, as differences between datasets would be expected to reduce, rather than inflate, spurious overlaps.

For each CHC class, we calculated the summed PIP values for SNPs located within DMRs and compared the observed values with a null distribution. To generate this null, we sampled 1000 random sets of 1 kbp methylation tiles (the same as used in methylome–environment association analyses), with each set matching the observed number of DMRs at a given *p*-value cut-off. For each random set, we identified all SNPs overlapping with the sampled tiles and recalculated the corresponding summed PIP value. In this way, we generated a null distribution that naturally incorporates variation in SNP numbers across 1 kbp tiles. We next estimated the empirical *p*-value by calculating the proportion of null sets that had summed PIPs greater than or equal to the observed values. To quantify effect size, we calculated the observed-to-null PIP ratios (i.e. the *x*-fold difference). We applied the same procedure to obtain the mean PIP values. These analyses were conducted both on all DMRs (genic and non-genic), as well as separately for DMRs' overlapping genes and individual genic compartments (exons, introns and DMRs overlapping both exonic and intronic regions, i.e. exon–intron junctions), with null sets sampled from the corresponding genomic context in each case.

In addition to the summed and mean PIP analyses, we tested whether DMRs tended to co-occur with SNPs with the strongest association to CHC variation, defined here as the top 5% of PIP values. This approach evaluates whether loci with the strongest associations to CHC variation are non-randomly distributed with respect to DMRs. To do so, we compared the observed number of SNPs with top 5% PIP values located within DMRs with a null distribution generated from 1000 permutations of randomly selected 1 kbp methylation tiles. Each sampling set at each permutation matched the number of DMRs at each *p*-value cut-off. We then calculated empirical *p*-values and *x*-fold differences as described above. These analyses were performed using all DMRs (genic and non-genic) and restricting the analyses to genic DMRs only.

To test whether the observed patterns could be explained by differences in minor-allele frequencies (MAFs) or SNP density within DMRs, we calculated the mean MAF and mean number of SNPs in the DMRs designated at each *p*-value cut-off. These observed values were then compared with null distributions generated using the same permutation approach described above. All statistics were computed separately for each CHC class and *p*-value cut-off. All statistical analyses were performed using R v4.3.2 [80].

(d) Association between high F_{ST} regions, cuticular hydrocarbons and single-nucleotide polymorphisms

Divergence in female CHC profiles has been previously shown to increase with genome-wide F_{ST} among *T. cristinae* populations, indicating that CHC divergence is associated with overall population genetic differentiation [71]. In parallel, prior work in this system has shown that ecotype-associated DMRs exhibit higher F_{ST} than genomic background levels, a pattern best explained by a balance between selection and gene flow [81]. Taken together, these findings raise the possibility that loci associated with CHC variation could co-vary with genomic regions of elevated genetic differentiation, as well as with DMRs. At the same time, regions contributing most strongly to elevated F_{ST} need not coincide with regions harbouring CHC-associated loci. To address this, we evaluated whether SNPs associated with each CHC class are associated with genomic regions showing elevated F_{ST} across population comparisons. This analysis tests for co-variation with genetic differentiation independently of the DMR results and thus evaluates whether any DMR–CHC association could be explained by shared associations with regions of elevated F_{ST}.

To evaluate co-variation with genetic differentiation, we estimated F_{ST} using whole-genome sequencing data originally published in [77] and reanalysed in [82], in which all data were aligned to the same reference genome used in the GWA for CHC variation and to estimate DMRs (v1.3c2) [74]. This dataset comprised 160 specimens from eight populations of both *Adenostoma* and *Ceanothus* ecotypes ($n = 20$ individuals per population; 28 pairwise comparisons; supplementary material, figure S1). We estimated F_{ST} following the methods described in [81]. Briefly, we calculated expected heterozygosity within populations (H_s) and total heterozygosity (H_t) using genotypic probabilities for each locus estimated in [82]. To facilitate comparison with the DMR results, we summarized F_{ST} in non-overlapping 1 kbp windows along the genome. For each pairwise population comparison, F_{ST} at each window was computed as a ratio-of-sums statistic, where $F_{ST} = \sum(H_t - H_s)/\sum(H_t)$.

For each pairwise population comparison, we defined highly differentiated windows as those in the upper 5% or 1% of the windowed F_{ST} distribution (i.e. above the 95^{th} or 99^{th} percentile, respectively). We then identified all GWA's SNPs falling within these windows and calculated both the summed and mean PIP values separately for each CHC class. To assess whether these observed values were greater than expected by chance, we generated null distributions by repeatedly sampling the same number of top F_{ST} windows at random, using a set of windows containing at least one GWA's SNP. For each random sample, we

recalculated the summed and the mean PIPs. We repeated this operation 1000 times and computed empirical *p*-values as the proportion of permutations with PIP values greater than or equal to the observed values and estimated the *x*-fold difference as described above. All tests were performed independently for each of the three CHC classes and on each of the 28 pairwise population comparisons to evaluate the consistency of effects across the several population pairs.

As additional controls, we also calculated the mean MAF and the number of GWA's SNPs in the top F_{ST} windows and compared these with null distributions generated using the same window-resampling procedure. As such, we here tested whether any observed signal could be explained by allele-frequency structure or SNP density, rather than by an association with F_{ST}. All analyses were implemented in R [80].

(e) Functional annotation of genes associated with cuticular hydrocarbon variation

Because our analyses revealed non-random co-variation between pentacosane-associated loci and ecotype-associated DMRs, we next examined the functional annotations of genomic regions where these two sets overlap, using gene ontology (GO) annotations. While functional annotation of the DMRs has been previously described in [74], the functional context of the CHC-associated loci remains less well characterized. To address this gap, we used the GO annotations from the *T. cristinae* reference genome (v1.3c2). We extracted the GO terms from predicted genes of the GFF3 annotation from [83] and overlapped the SNPs used in the GWA with the predicted genes. As a result, we obtained GO terms for each gene where we had SNPs with PIP values.

To test for functional enrichment, we performed GO term enrichment analyses using TopGO v2.46 [84] package in R [80]. Given the polygenic architecture of the CHCs, we defined SNPs most associated with each CHC class as the top 5% of PIP values. For each CHC class, we tested whether GO terms associated with these top SNPs were over-represented relative to genomic background levels. To this end, we used Fisher's exact test, followed by Benjamini–Hochberg false discovery rate correction (FDR) of 10%. FDR was estimated separately for GO terms classified as biological processes, cellular components and molecular function, classified by TopGO.

Because significant co-variation with DMRs was observed only for pentacosane-associated loci (see §3), we next examined the functional annotations of genes overlapping DMRs that contained SNPs in the top 5% PIP values for pentacosane variation (supplementary material, table S9). For each gene, we compiled the GO terms across the different DMR *p*-value cut-offs, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations, obtained using the EggNOG-mapper (emapper.py) pipeline with the Diamond database [85]. In addition, we extracted protein sequences from these genes based on the GFF3 annotation using the AGAT utility agat_sp_extract_sequences.pl [86]. These protein sequences were queried against the UniProtKB/Swiss-Prot database using BLASTp [87] and the best hit was retained based on the lowest *e*-value (if below 1×10^{-5}).

3. Results

(a) Differentially methylated regions co-vary with pentacosane-associated loci

Consistent with our predictions, we found that DMRs exhibited higher overall levels of association with pentacosane-associated loci, whereas no comparable signal was detected for other CHC classes. Specifically, SNPs associated with female pentacosanes exhibited significantly higher summed PIP values within DMRs than null expectations (approx. 2- to 7-fold higher, $p < 0.01$ in all comparisons; figure 3). This result was robust across all *p*-value cut-offs (table 1; supplementary material, table S2) and was also observed when considering the mean PIPs for SNPs within DMRs (supplementary material, table S3). Similar patterns were obtained when restricting the analyses to genic DMRs only (supplementary material, tables S4 and S5). Stratifying DMRs by genic context further indicated that this pattern was not specific to a single genic functional compartment. That is, both mean and summed PIPs tended to be higher than expected by chance in DMRs overlapping exons, introns and exon–intron junctions, with effect sizes of comparable magnitude across contexts (supplementary material, tables S6 and S7).

Consistent with these results, DMRs defined by more relaxed *p*-value cut-offs ($p < 0.0061$ and $p < 0.01$) showed significant overlap with SNPs in the top 5% of PIPs for pentacosanes (2.7-fold relative to null expectations; $p < 0.01$). This pattern was observed for both DMRs and those located within genes, whereas more stringent *p*-value cut-offs did not yield significant overlap (0.5–1.5-fold; $p > 0.10$; supplementary material, tables S8 and S9). Importantly, we found no significant differences in either the number or the mean MAFs of SNPs within DMRs compared with null distributions in any analysis (table 1; supplementary material, tables S3, S5, S7), indicating that the observed results were unlikely to be driven by biases in SNP density or allele frequencies.

By contrast, DMRs showed no significant association with SNPs most associated with female heptacosanes or nonacosanes in any analysis, whether considering all DMRs or DMRs in different genomic contexts (table 1; supplementary material, tables S3–S5 and S8–S13). For example, the sum of PIPs for SNPs associated with hetpacosanes within DMRs was 0.6–0.8-fold lower than expected by chance ($p > 0.10$) and 0.3–1.2-fold in DMRs' SNPs associated with nonacosanes ($p > 0.10$; table 1). Therefore, the correspondence between ecotype-associated DMRs and CHC-associated loci appears to be specific to pentacosanes.

(b) Cuticular hydrocarbon-associated single-nucleotide polymorphisms do not co-vary with highly genetically differentiated genomic regions

In contrast to the co-variation observed between pentacosane-associated loci and ecotype-associated DMRs, we found no evidence of co-variation between SNPs associated with any CHC class and regions of high genetic differentiation (supplementary

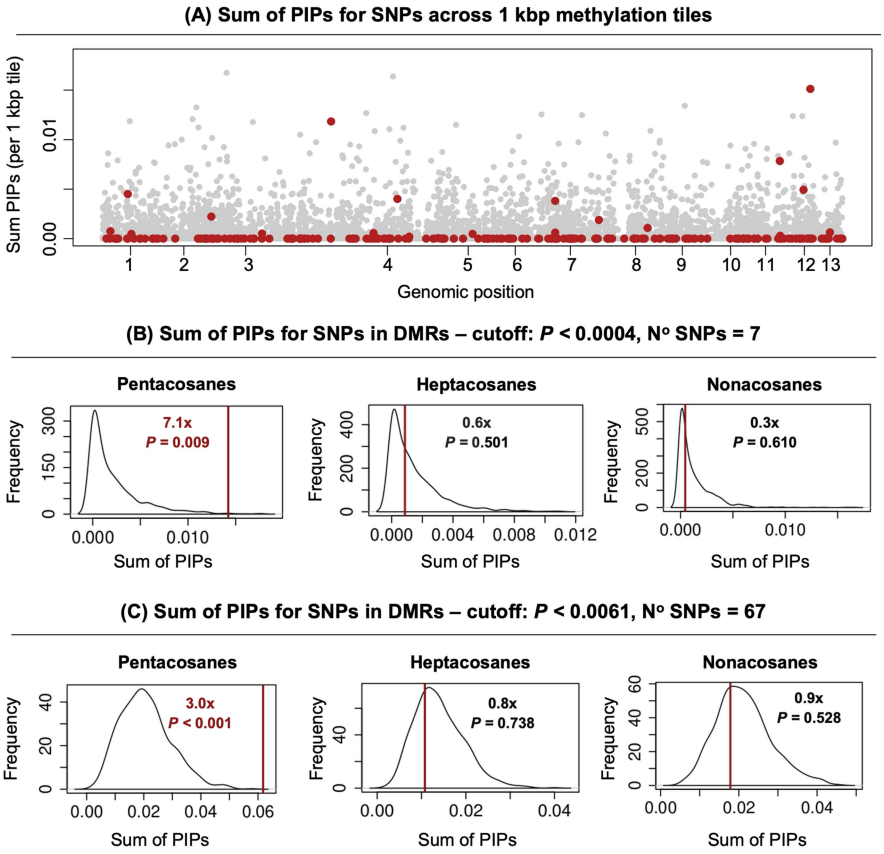

Figure 3. Sum of PIP values of SNPs associated with female CHC variation in DMRs compared with genomic background expectations. (A) Sum of PIPs for pentacosanes across 1 kilobasepair (kbp) methylation tiles, with DMRs highlighted in red. In this graph, DMRs were delimited by the $p < 0.0061$ threshold. The great majority of 1 kbp methylation tiles (98%) showed summed PIPs < 0.001. The graphs in (B) and (C) show the sum of PIPs for SNPs associated with the three CHC classes (i.e. pentacosanes, heptacosanes and nonacosanes) falling within DMRs (red bars). These observed values are compared with a null distribution generated through randomizations. Each plot shows the results for a different *p*-value cut-off used to delimit DMRs in the methylome–environment association analyses (MACAU) [74,76], here represented by $p < 0.0004$ and $p < 0.0061$ cut-offs (0.04th and 0.40th percentile of the empirical *p*-value distribution from MACAU; table 1). The results consistently show that DMRs co-vary with loci associated with female pentacosane variation. x-fold difference and *p*-values for these results are shown inside each graph.

material, tables S14–S17). Across the different population comparisons, windows in the upper 5% of the F_{ST} distribution had mean PIP values comparable to genome-wide background levels for all CHC classes (1.0-fold difference, all $p > 0.10$) and slightly lower summed PIP values on average (0.9-fold, all $p > 0.10$; supplementary material, tables S14 and S15). In only three of the 28 pairwise comparisons, windows with high F_{ST} exhibited higher MAF than expected by chance (with $p < 0.05$) and no comparison contained more SNPs in the windows than expected at background levels ($p > 0.10$; supplementary material, table S18). We observed similar patterns using more stringent thresholds to define high genetic differentiation (i.e. 1% F_{ST} windows; supplementary material, tables S16 and S17).

(c) Functional enrichment patterns differ among cuticular hydrocarbon classes

We detected several functional terms that were enriched among genes with SNPs most strongly associated with CHC variation. With the 10% FDR correction, we found that SNPs associated with pentacosane and nonacosane variation were enriched for GO terms related to cell population proliferation ($q < 0.10$), whereas heptacosanes showed enrichment for extracellular matrix containing collagen and response to ecdysone ($q < 0.10$; supplementary material, table S19). Additional GO terms showed enrichment across CHC classes but did not remain significant after FDR correction. For example, pentacosanes were marginally enriched for GO terms associated with cell division functions; heptacosanes with basement membrane (extracellular matrix that forms the supporting structure on which epithelial cells grow) and response to ecdysone; and nonacosanes with cell division, UV protection and heat shock protein binding (supplementary material, table S19).

Genes overlapping DMRs that contained SNPs in the top 5% PIP for pentacosanes were functionally heterogeneous, but showed functions for pathways related to hormonal metabolism and signal transduction (supplementary material, table S20). Across multiple DMR *p*-value cut-offs, we identified a gene that encodes juvenile hormone esterase—an insect enzyme involved in the degradation of juvenile hormone and regulation of moulting and reproduction. Other genes included components of conserved signalling cascades, such as mitogen-activated protein kinase 3 (MAPK3) and transforming growth factor beta-1-induced transcript 1 (TGFB1I1), which are associated with kinase activity, cytoskeletal regulation and growth factor-mediated signalling. Other loci included a protein phosphatase 1 gene and a retrovirus-related protein, although functional annotations for these genes were more limited (supplementary material, table S20).

Table 1. Magnitude of the ratio between the summed PIPs for CHC-associated SNPs within DMRs compared with null expectations (i.e. *x*-fold), considering DMRs designated by different *p*-value cut-offs.

cut-off	CHC	♀ penta	♀ hepta	♀ nona
$p < 0.0004$	observed sum	0.0142	0.0009	0.0004
	expected sum	0.0020	0.0016	0.0014
	x-fold	**7.1**	0.6	0.3
	p	**0.009** **	0.501	0.610
	p SNP number	0.313	0.324	0.317
	p MAF	0.833	0.849	0.836
$p < 0.0028$	observed sum	0.0383	0.0054	0.0080
	expected sum	0.0106	0.0073	0.0068
	x-fold	**3.6**	0.7	1.2
	p	**0.001** **	0.639	0.324
	p SNP number	0.154	0.118	0.140
	p MAF	0.709	0.683	0.711
$p < 0.0061$	observed sum	0.0618	0.0108	0.0125
	expected sum	0.0204	0.0144	0.0133
	x-fold	**3.0**	0.8	0.9
	p	**<0.001** **	0.738	0.528
	p SNP number	0.111	0.109	0.104
	p MAF	0.792	0.777	0.784
$p < 0.01$	observed sum	0.0717	0.0179	0.0178
	expected sum	0.0325	0.0232	0.0213
	x-fold	**2.2**	0.8	0.8
	p	**0.004** **	0.761	0.664
	p SNP number	0.335	0.316	0.321
	p MAF	0.771	0.775	0.758

♀ penta = female pentacosanes; ♀ hepta = female heptacosanes; ♀ nona = female nonacosanes and MAF = minor-allele frequency. * $p < 0.05$; ** $p < 0.01$.

4. Discussion

Signalling traits such as visual displays, acoustic cues and chemical signals can affect mate choice and be shaped by sexual selection. Divergence in signalling traits can reduce gene flow between populations, contribute to reproductive isolation and help maintain species boundaries [88,89]. Although models of sexual selection commonly assume a direct relationship between genotype and phenotype, both signalling trait variation and preferences can vary with environmental conditions [30,34,90]. In this context, investigating epigenetic marks associated with signal traits may help clarify how environmentally associated variation in these traits arises. Here, we contribute to this effort by showing that genomic regions associated with a key sexual signalling trait (pentacosanes) exhibited non-random co-variation with regions exhibiting ecotype-associated DNA methylation patterns.

Across all metrics evaluated, DMRs showed consistently elevated associations with pentacosane-associated loci. For instance, the summed PIPs of pentacosane-associated SNPs located within DMRs were 2- to 7-fold higher than expected under null expectations, a pattern that was also observed for genic DMRs. Within genes, summed and mean PIPs were comparable across exons, introns and exon–intron junctions. This suggests that the observed co-variation was not driven by a single functional compartment, but rather seems to involve the entire gene body. By contrast, we detected no co-variation between DMRs and loci associated with heptacosanes or nonacosanes under any metric.

Our overall results show that DMRs co-vary with SNPs most associated with variation in pentacosanes—the CHC class known to play an important role in mate choice and sexual isolation in *T. cristinae* [71]. Here, such co-variation reflects a non-random genomic correspondence, where loci associated with pentacosane variation tend to occur within regions showing ecotype-associated differential methylation more often, or with greater association strength, than expected by chance. Most critically, we found no such evidence for hepta- or nonacosanes, despite their polygenic architecture and variation with host-plant ecotype. These findings suggest that co-variation with DMRs is not a general property of loci associated with CHC variation, but is restricted to those associated with the CHC class most strongly implicated in sexual signalling, rather than those more broadly associated with climatic variation (figure 2D) [71,73].

One possible explanation for the observed co-variation between ecotype-associated DMRs and pentacosane-associated loci is that both would reflect underlying population genetic differentiation. However, at the genomic scale at which DMRs were defined (1 kbp windows), we found no evidence that regions of elevated F_{ST} co-varied with any CHC class, including pentacosanes. This indicates that the observed co-variation between DMRs and pentacosane-associated loci is not driven by generalized population divergence or local peaks of genetic differentiation. Instead, it is consistent with DNA methylation co-occurring with the polygenic set of loci associated with pentacosane variation. Under this scenario, genetic divergence may arise through the cumulative effects of many small-effect loci, such that it does not need to be accompanied by elevated local F_{ST} peaks, even when pentacosane divergence co-varies with genome-wide F_{ST} among populations [69].

Having established that the observed co-variation with DMRs is specific to pentacosanes and not explained by genetic differentiation, we next assessed the functions of DMRs and pentacosane-associated loci. Previous work has shown that ecotype-associated DMRs are enriched for functions related to protein metabolism and membrane processes, especially signal transduction and transmembrane transport activity [74]. Interestingly, these membrane-related functions (e.g. membrane, transmembrane transport, and ion transport) are also enriched in genomic regions showing parallel evolution to host plants in *T. cristinae* [77], suggesting that these processes play an important role in host-plant use. In this study, we estimated functional annotations associated with each CHC class (see supplementary material for details) and examined the overlap between DMRs and loci most strongly associated with pentacosanes. Although the number of genes underlying this overlap was limited, the functions represented included pathways related to hormone metabolism and conserved intracellular signalling cascades, such as mitogen-activated protein kinase (MAPK) and growth factor-mediated processes. While the precise mechanistic links and phenotypic consequences of this overlap remain to be elucidated, our results highlight regulatory pathways that may contribute to CHC variation and possible modulation by methylation.

Considering our collective results, we raise several non-mutually exclusive scenarios that are consistent with the observed co-variation between ecotype-associated DMRs and pentacosane-associated loci. DNA methylation patterns may be genetically determined and tied to specific SNPs that affect pentacosane variation, acting as a regulatory layer on *cis*-genetic effects [52,91]. Alternatively, methylation differences may be induced by host-plant habitat and influence pentacosane expression [92,93], or they may arise through genotype-by-environment interactions, where different genotypes respond epigenetically to distinct host-plant habitats [40,94]. Across these scenarios, DNA methylation could co-vary with pentacosane-associated loci potentially through effects on gene regulation, without requiring methylation itself to be environmentally induced. Moreover, in *T. cristinae*, gene-body methylation is positively associated with gene expression [74]. As such, because the associations we detected were primarily driven by genic DMRs, the observed co-variation is consistent with methylation being associated with regulatory regions relevant to pentacosane variation. Distinguishing among the scenarios raised above will require future studies integrating individual-level genotype, methylation, and phenotypic data.

Beyond mechanistic links between DNA methylation and CHC variation, our results raise broader questions about how epigenetic variation may contribute to sexual isolation in *T. cristinae*. Because pentacosanes are involved in ecotype-specific sexual isolation in *T. cristinae* [70,71], DNA methylation could potentially contribute to this process. However, the extent of this contribution probably depends on the ecological and evolutionary context in which each ecotype occurs. Although *T. cristinae* host-plant ecotypes are biologically distinct and differ in multiple traits (including CHC profiles), the degree of sexual isolation between them varies widely [66]. This variation probably reflects a mosaic of factors, including a balance of selection and gene flow (e.g. driven by geographical and climatic differences) and additional traits influencing mate choice beyond CHCs [66,71,95]. Adding to this complexity, previous work shows that DMRs are associated with genetic differentiation among populations, but not specifically with host-plant use [81]. As such, any potential contribution of DNA methylation to sexual isolation is likely to be context-dependent and intertwined with multiple ecological and evolutionary processes.

Despite the observed co-variation between ecotype-associated DNA methylation patterns and loci associated with pentacosane variation, our results do not establish a causal relationship between methylation and signal trait variation. To build on these findings, future work should directly test whether variation in DNA methylation contributes to differences in pentacosane expression. This could involve experimental or statistical approaches that link DNA methylation levels at DMRs to variation in CHC profiles across individuals with differing genotypes (e.g. through manipulation of DNA methylation and testing for CHC response). In addition, identifying the environmental factors that may trigger methylation changes (e.g. host-plant species) will be a key to understanding whether DNA methylation is responsive to environmental change and to what extent it is determined by genetic variation. If a causal relationship between DNA methylation and pentacosane expression were to be established, an important next step would be to test whether DNA methylation is associated with differences in mate choice and reproductive success. Such work would help clarify the functional significance of DNA methylation for sexual signalling and assess whether methylation contributes to phenotypic plasticity in sexual selection, including condition-dependent signalling.

Our study is one of the first to establish a link between epigenetic variation and a signal trait of mate choice, despite the limitations on causal inference. This connection offers a promising avenue to understand the molecular mechanisms through which environmental variation may influence signalling traits and mate choice, and how these effects vary across space and time. This dynamic view of sexual selection challenges traditional models that assume immutable sexual selection and instead supports a framework in which signal expression and mate preferences may be more labile [30,90,96]. In addition, sexual selection on plastic traits could, in theory, increase the rate and extent of local adaptation and promote population divergence [97]. Our work thus ultimately contributes to a growing understanding of how environmentally responsive sexual selection, mediated by regulatory and physiological variation in signalling traits, may influence reproductive isolation and divergence in the wild [16,98–100].

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Data availability: this manuscript does not contain new data, all cited material is publicly archived as described in §3. All scripts and code used for analysis has been archived at Zenodo [101].

Supplementary material is available online [102].

Declaration of AI use. I, Clarissa de Carvalho, used Grammarly to correct for grammar and typos to improve readability.

Authors' contributions. C.F.d.C.: conceptualization, formal analysis, investigation, methodology, writing—original draft, writing—review and editing; N.P.P.: writing—review and editing; J.S.: writing—review and editing; R.R.: writing—review and editing; Z.G.: conceptualization, formal analysis, investigation, writing—review and editing; P.N.: conceptualization, investigation, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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