DNA methylation patterns respond to thermal stress in the viviparous cockroach *Diploptera punctata*

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Abstract

It is increasingly recognized that epigenetic mechanisms play a key role in acclimatisation and adaptation to thermal stress in invertebrates. DNA methylation and its response to temperature variation has been poorly studied in insects. Here, we investigated DNA methylation and hydroxymethylation patterns in the viviparous cockroach *Diploptera punctata* at a global and gene-specific level to variations in temperature. We specifically studied methylation percentage in the heat shock protein 70 (Hsp70),whose function is linked to thermal plasticity and resistance. We found high levels of DNA methylation in several tissues but only low levels of DNA hydroxymethylation in the brain. Hsp70 methylation patterns showed significant differences in response to temperature. We further found that global DNA variation was considerably lower at 28°C compared to higher or lower temperatures, which may be indicative of the optimal temperature for this species. Our results demonstrate that DNA methylation could provide a mechanism for insects to dynamically respond to changing temperature conditions in their environment.

Introduction

Epigenetic processes are central to trait evolution because novel phenotypes may be generated in response to environmental cues. This process promotes differences in gene expression, and therefore, it might allow acclimatisation to environmental changes and even enhance local adaptation [32, 17]. Further, under novel or environmental conditions, epigenetic variation may increase [50]. This epigenetic variation may contribute to heritable variation on which selection can act or create novel selectable

phenotypes. Such novel phenotypes may be beneficial if the environment is constant across generations.

Among the main epigenetic processes, DNA methylation is the best studied epigenetic mark, which involves the addition of a methyl group on the fifth position of the cytosine. In mammals, DNA methylation is mainly enriched in regulatory regions and is associated with gene silencing [8, 16]. By contrast, in invertebrates DNA methylation is enriched in exons and is associated with gene activation . DNA methylation is highly dynamic as it can vary in response to, for example, environmental factors, requirements of the cell, or developmental stage [22]. DNA hydroxymethylation (DNA 5-hmC), by contrast, is a largely unexplored epigenetic mechanism that is presumably involved in gene upregulation and active demethylation processes [35].DNA hydroxymethylation is mainly found in the nervous system, suggesting important and specific neural and developmental functionality [45, 40].

To date, DNA methylation patterns in response to environmental stressors such as thermal stress have been poorly studied, especially in insects. Because of the significant relevance that methylation may have for adaptation to environmental stressors we sought to establish how this key epigenetic mechanism is affected by arguably the most important abiotic stressor currently, temperature.

To investigate the effects of thermal stress on methylation we used two different approaches. First, we focussed on one of the main genes involved in thermal response, the Heat Shock Protein 70 (Hsp70). Apart from performing several important physiological roles, such as secretion, degradation and regulation, this protein also facilitates organismal thermotolerance [30, 29]. For example, in the fruit fly, thermotolerance varies

according to the amount of Hsp70 present in the cell before heat stress. Usually, Hsp70 will be absent if a cell, or an organism, has not been exposed to thermal stressors [30]. Second, we analyzed methylation patterns and methylation variation, on a global scale. Several studies propose that methylation profiles are determined by the environment and that individuals living in similar environmental conditions will have similar methylation profiles [23, 43, 48]. It has been suggested that high levels of epigenetic variation could help to overcome reduced levels of genetic variation or abrupt changes in the environment, by inducing phenotypic changes that might help organisms to survive in exotic environments [5].

It is important to emphasize that the way that methylation may respond to the environment could also be influenced by the genotype. Even though methylation patterns can be determined by the environment and behave as an autonomous system [36], several studies have shown that methylation patterns can also be determined by the genotype. This close link between the genotype and methylation patterns is possible because the latter may have originated from silencing transposable elements or random epimutations [34, 14, 38]. Notably, in a variety of organisms collected from the wild, a higher degree of epigenetic variation compared to genetic variation has been recorded [12, 18].

In this paper, we present the first investigation of DNA methylation and hydroxymethylation in the cockroach Diploptera punctata and its response to thermal stress. Diploptera punctata is among the very few truly viviparous insects and the only truly viviparous cockroach. It belongs to Blattodea, a group that presents several adaptations to thermal stress [7]. Our first research aim was to investigate if DNA methylation and hydroxymethylation are present in Diploptera punctata. To achieve this, we

quantified global methylation and hydroxymethylation levels at a tissue-specific level. Then we sought to establish if methylation patterns are affected by temperature and if organisms sharing similar genotypes will react similarly to thermal stress. This lead to our second research aim, which was to study if methylation of Hsp70 was affected by temperature. We investigated the intragenic region of the Hsp70 gene after we exposed seven genotypes to four different temperature treatments. Our final research aim was to investigate how global methylation patterns respond to thermal stress. To achieve our final aim we use MS-AFLPs to analyse methylation patterns in individuals from seven different genotypes that were exposed to four different temperature treatments.

Methods

0.1 Animal maintenance

D. punctata colonies had been established for over 10 years in the laboratory and were maintained in plastic containers ($30 \times 22 \times 20$ cm) at 25°C on a 12:12h light: dark cycle and fed with blended dog food (WAGG Complete Dog Food) and water provided ad libitum.

0.2 Global DNA Methylation percentage

Dissections Cockroaches were dissected in bath saline solution (135 Mm NaCl, 5 mM KCl, 4mM $MgCl_2$ $6H_2O$, 2mM $CaCl_2$ $2H_2O$, 5 mM TES, 36 mM sucrose). We dissected the legs, head, fat body and embryos. Embryos were obtained between days 45 and 55 of pregnancy (59-75% of total development [47]). At this stage, the embryos are between 4 and 5 mm long.

Tissues were immediately frozen in dry ice and stored at -80°C until used.

DNA extraction and methylation analysis DNA was extracted from adult females using Qiagen DNeasy Blood & Tissue Kit following the manufacturer's protocol. All DNA samples were precipitated and cleaned using standard ethanol precipitation [44]. After DNA extraction, all samples were quantified using Qubit dsDNA HS Assay Kit (ThermoFisher). Methylation and hydroxymethylation global levels were quantified using the MethylFlash Methylated DNA 5-mC Quantification Kit (Colorimetric), and the MethylFlash Hydroxymethylated DNA 5-hmC Quantification Kit (Colorimetric). We used 100 ng of DNA as input and followed the protocol as indicated by the manufacturer (Epigentek). Every sample was done in duplicate. For negative control in both assays we used 100 ng of adult *Drosophila melanogaster* DNA, as the *Drosophila* genome does not carry DNA methylation or hydroxymethylation [9, 49]. Exclusively for the hydroxymethylation assay, we used mouse brain as a positive control because hydroxymethylation levels within the mouse brain is reported to be 0.2%[45].

Statistical analysis All data were analysed using linear mixed-effects models in the R environment [41], using the packages lme4 and car [19, 6]. The logarithm of global methylation and hydroxymethylation levels were used as response variables, tissue (five levels and six levels, respectively) was a fixed factor and individual considered as a random effect.

Temperature manipulation

We isolated pregnant females (parental generation) and monitored them daily until they gave birth to the first generation (F1). We used the first two clutches from the parental generation. During the first week of life, we randomly allocated individuals from the F1 to four different temperature regimes: 26°C, 28°C, 30°C and 32°C. To control the temperature we constructed wooden boxes (62 x 40 x 37 cm) and added a ceramic heating element (Exo-Terra), a thermostat, (600 watts, Habitat) a thermometer (Exo-Terra), a humidity meter, and LED light programmed to 12/12 hrs light cycle. Inside the boxes, we kept the cockroaches in plastic boxes (15 x 7 x 15 cm) grouped by family. Water and blended dog food (WAGGS) was provided once weekly. We monitored the cockroaches weekly and measured (Mitutoyo calliper), weighed, and marked any new adult.

DNA extraction Once the cockroaches had reached adulthood and gave birth to the next generation (F2) we sacrificed adult individuals using liquid nitrogen. We dissected the head in sterile conditions under a UV hood. Tissue was stored at -80°C before usage. We homogenized tissue manually using plastic pellets and extracted DNA using Qiagen tissue and blood extraction kit as indicated by the manufacturer. The cockroach eye pigment precipitates with the DNA and inhibits PCR. To avoid PCR inhibition we cleaned the samples using the Qiagen cleaning kit as indicated by the manufacturer.

Response of Hsp70 gene methylation to thermal stress

Amplification of Hsp70 gene in *Diploptera punctata* To obtain the Hsp70 gene body DNA sequence of *Diploptera punctata*, we first collected

Hsp70 available sequences from closely related species. In total we collected three sequences from three species: *Blatella germanica* (Accession No: PYGN01000002.1:4236897-4238334), *Periplaneta americana* (Accession No: KY661334.1) and *Cryptocercus punctulatus* (Accession No: JQ686949.1). The sequences were aligned on Clustal Omega [11]. We then used PriFi [20] (https://services.birc.au.dk/prifi/main.py) to design multiple set of primers. This tool is useful for designing primers from multiple sequence alignments derived from phylogenetically related species, in particular when working with organisms without a reference genome. Two different parameters were entered into the system for Hsp70 alignment. The sequence of the primers that amplified successfully the desired fragment is: Fw 5'-AAGGGTCATGGAGAACGCAA-3' and Rv 5'-CTCTTCATGTTGAAGCAGTA-3'. For the PCR amplification, we added 2µl (150ng/µl) of DNA to the PCR mix (Foward primer 1µl (0.4µM), Reverse primer 1µl (0.4µM), PCR mix 12.5µl, Nuclease free water 8.5µl) with the following PCR conditions: 95 °C for 4 min, followed by 35 cycles of 95 °C for 15 sec, 61 °C for 1 min, 72 °C for 1.15 min and 72 °C for 10 min.

To verify that the amplified section corresponds to Hsp70 we performed Sanger sequencing. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and the purified fragments were sent as premixed samples to the Genomic Technologies Core Facility (GTCF, University of Manchester) for Sanger sequencing. The samples contained either forward or reverse primers, purified templates and nuclease-free water to make a total 10µl reaction volume.

Bisulfite conversion and pyrosequencing DNA was bisulphite converted using the PyroMark PCR Kit (Qiagen) with 200ng. We aligned

Diploptera punctata Hsp70 sequence with the Hsp70 sequence of *B. glabratahe*. In the conserved regions we identified, and selected for further analysis, a region with four CpGs that was found to be methylated in *B. glabratahe* [29]. We used PyroMark Assay Design software (Qiagen) to design a set of a forward, reverse and sequencing primers, which are as follow: Fw 5'-ATTTAAGTTTAAGAAGGTGAGAGAGTAATG-3', Rv 5'-CTCCTTTCCTATTAATTTTTCAACTACTA -3', sequence 5'-GGTGTTTTATAAATTGAGGTTATT-3'. The reverse primer is biotinylated at 5' end. The PyroMark Q24 (Qiagen) was used for pyrosequencing using PyroMark Q24 Advanced Reagents kit (Qiagen). From the PCR sample 10µl was used for each pyrosequencing reaction using the sequencing primer 5'- TTGTTGGTGGTAGTTTT-3' and were performed in duplicate. PyroMark PCR Kit (Qiagen) was used to carry out methylation-specific PCRs as specified by the manufacturer.

Statistical methods All four positions were analyzed independently. Data were analysed using linear mixed-effects models in the R environment [41], using the packages lme4 and car [19, 6]. The percentage of methylation at each cytosine was used as a response variable, the genotype (six levels), temperature (four levels) and developmental time were considered as a fixed factor.

Response of global methylation patterns to thermal stress

Methylation sensitive amplified length polymorphisms DNA extraction was done as described above. MspI and HpaII are isoenzymes with the same restriction site (CCGG) but with different sensitivities to DNA methylation [1, 18]. HpaII activity is blocked when the inner or outer C

is methylated at both strands. By contrast, MspI cleavage is not allowed when the outer cytosine is fully methylated. By treating DNA with both enzymes we can identify four different methylation states at each restriction site (Type 1: when both enzymes cut (no methylation) Type 2: when HpaII cleavage is blocked and MspI does cut (methylation present in the internal cytosine) Type 3: when HpaII does cut and MspI activity is blocked (hemimethylated outer C) Type 4: Both of the enzyme activity is blocked (hypermethylation or sequence mutation at the restriction site).

For MS-AFLPS a total of 67 organisms were screened. All samples were processed in duplicate. We followed the protocol in Amarasinghe et al (2014) [1], with some modifications (see primers in table S5). We digested DNA in two separate reactions. The first one used EcoRI (0.05µl NEB, 20 000 units/ml) + MspI (0.025µl NEB, 20 000 units/ml). The second one used EcoRI + HpaII (0.5µl NEB, 20 000 units/ml). We added 5µl of DNA to the two independent digestion mixtures (EcoRI, MspI/HpaII, 1µl NEB cut buffer 10X, and 3µl of ddH_2O). The reaction was incubated for three hours at 37 °C. Immediately after digestion, we added 5µl of the digested product to the ligation reaction (0.25µl T4 DNA ligase NEB (400000 units/ml), 1 µl of NEB ligase buffer, 1µl of EcoRI adapter (5 pmol), 1µl HpaII-MspI (50 pmol), and 1.75 ul of ddH_2O). The ligation reaction was incubated at 37 °C for three hours and left overnight at room temperature. Then, we ran a pre-selective PCR (pPCR) by adding 5µl of the ligated product to the pPCR mix (1.25 µl pre-selective EcoRI primers (0.5 µM), 1.25µl pre-selective HpaII-MspI primers (0.5 µM), 12.5 µl of PCR master mix Agilent, Paq5000 Hotstart PCR Master Mix) and the following PCR conditions: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 58 °C for 1 min, 72 °C for 1 min and 72 °C for 5 min. Then we ran selective PCR (sPCR) in which

three different primers were used. We used 5µl of the pPCR product as a DNA template, which was added to the following mix (0.5 µl selective EcoRI primer (0.5µM), 0.5µl of selective HpaII/MspI primers (0.5µM) and 5µl of PCR master mix (Agilent, Paq5000 Hotstart PCR Master Mix), with the following PCR conditions: 94 °C for 2 min, followed by 13 cycles of 94 °C for 30 sec, 65 °C (decreasing 0.7 °C per cycle) for 30 sec, followed by 30 cycles of 94 °C for 30 sec, 56 °C for 30 sec, 72 °C for 1 min, and a final extension of 72 °C for 5 min. Each of the forward selective primers was marked with a different fluorophore (6-FAM, HEX, ROX). The fluorophore allows the identification of the fragment after capillary electrophoresis. Finally, we mixed 0.5µl of the sPCR product with 0.4µl of 500 LIZ dye Size Standard (ThermoFisher) and 9µl of Hi-Di formamide (ThermoFisher). The samples were then sent to the University of Manchester sequencing facility for fragment analysis.

Statistical analysis

Effect of temperature and family on global methylation patterns We performed a perMANOVA (Permutational multivariate analysis of variance; [2]) using the adonis function from the vegan R package [37] to test the effect of temperature, family and maternal developmental temperature on methylation patterns. We set the permutation number at 1,000,000 and used the Euclidean method to create the distance matrix, which was used as the response variable. We also performed a pairwise perMANOVA using pairwiseAdonis function in R [3]. The first and second generation were analysed independently. For the first generation, we considered temperature and family as predictors. For the second generation, we considered temperature, family and maternal developmental temperature as predic-

tors. However, for the pairwise analysis, in both generations, we tested for temperature effects and controlled for family and vice-versa.

Methylation variation To determine whether the variation in methylation patterns is influenced by temperature we obtained the distance of each coordinate from the PCoA to the center of each respective group using the Euclidean distance formula:

$$D = \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2} \quad (1)$$

where D is the distance between the centroid and a given point, x_1 and y_1 are the coordinates of interest and x_2 and y_2 are the centroid coordinates.

To determine whether the observed differences in methylation distance between temperature groups were greater than we would expect to see by chance alone, and therefore statistically significant, we ran a series of pairwise comparisons of the different developmental temperatures (e.g. 26°C vs 28°C, 26°C vs 30°C, etc) and compared them to a null distribution of differences obtained in 10^6 randomly generated permutations of the data. We created permutations by reassigning the observed data points between the temperature groups, subject to the constraint that the number of observations could not change within temperature groups. This constraint is important because there are different numbers of observations in the different temperature groups, and the variance of methylation per temperature group depends on the number of observations. For each comparison, the *p-value* is the proportion of the null distribution in which the difference between temperatures was as great as, or greater than, in the observed data.

Methylation proportion We obtained the proportion of methylated sites per sample by counting the number of methylated sites and dividing by the total number of loci. We tested whether family and temperature had an effect on methylation proportion using a linear model with the logarithm of methylation proportion as the response variable and family and temperature as predictors. We also tested if methylation proportion has an effect on phenotypic traits such as metabolic rate, developmental time and weight. The phenotype data is taken from the individuals described in chapter 1. For this, we used the phenotype as a response variable and methylation proportion, developmental temperature and sex as predictors.

Genetic and epigenetic correlation To analyze the correlation between epigenetic variation and genetic variation we performed a Mantel test using the R vegan package [37]. The Mantel test is a correlation between entries of two dissimilarity matrices. To run the Mantel test we first created two different distance matrices using the Euclidean method. We created the first matrix using the MSL and another using NML for both generations separately. For the Mantel test, we used the Pearson method and ran 1,000,000 permutations.

Results

Global methylation levels

We found evidence of DNA methylation in all tissues (average DNA methylation level 8.8%), with no significant difference among them (GLM $F_{3,25} = 0.9535, p > 0.05$). We used adult *Drosophila melanogaster* as a negative control, (no DNA methylation), and, as expected we did not find

evidence of methylation in *Drosophila melanogaster*. Further, all *Diploptera punctata* tissues showed significantly higher levels than *Drosophila* (HSD, *Drosophila* vs embryo, $t-ratio = -9.81, p < 0.0001$, *Drosophila* vs fat body,$t-ratio = -7.71, p < 0.0001$, *Drosophila* vs head, $t-ratio = -9.71, p < 0.001$, *Drosophila* vs leg, $t-ratio = -9.75, p < 0.001$;Fig. 1 **A**). In contrast to DNA methylation, global DNA hydroxymethylation was found only in the cockroach head. We found that 0.75% of the cytosines were hydroxymethylated (Fig. 1 **B**). For the hydroxymethylation assay we also used adult individuals of *Drosophila melanogaster* as a negative control, and mouse brain as a positive control. Interestingly, we found similar levels of 5-hmCs in the head of *D. punctata* as those found in the mouse brain (0.22%). The levels of hydroxymethylation in the mouse brain and in the cockroach head were not significantly different from each other ($z-value = -1.51, p > 0.05$). The hydroxymethylation levels of *Drosophila* were not different from the levels of the embryo, fat body and leg (*Drosophila* vs embryo $z-value = 0.123, p > 0.05$ *Drosophila* vs fat body $zvalue = 0.79, p > 0.05$, *Drosophila* vs leg $z-value = 1.29, p > 0.05$).

Response of Hsp70 gene methylation to thermal stress

We analyzed four cytocines in the intergenic region of the Hsp70. The four positions analyzed were highly methylated (98.806%, 94.838%, 93.774% and 88.483% in the first, second, third and fourth positions respectively). All four CpGs analyzed showed significant temperature effects on methylation (Postion 1: $F_{3,20} = 4.575, p = 0.013$; Position 2: $F_{3,20} = 12.50, p = 7.91e-05$; Position 3: $F_{3,20} = 5.986, p = 0.004$; Position 4: $F_{3,20} = 9.048, p = 0.0005$). Family had an effect on methylation percentage at three of the four positions analyzed (Postion 1: $F_{6,20} = 3.922, p = 0.009$; Position 2:

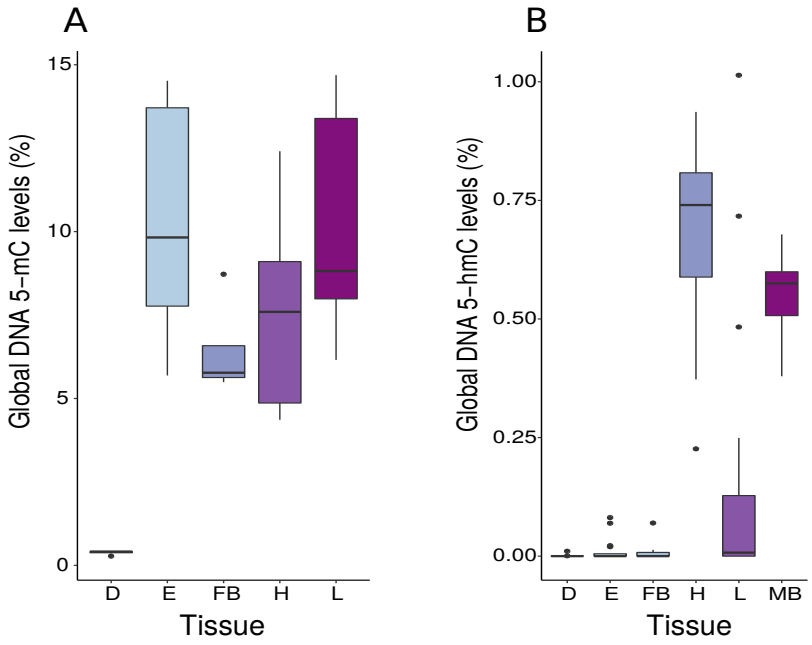

Figure 1. Methylation and hydroxymethylation levels in *Diploptera punctata*. **A**. Global methylation levels from four different tissues (embryo (E), fat body (FB), head (H), and legs (L)). *Drosophila* DNA (D) was used as a negative control (no DNA methylation). **B**. Global DNA hydroxymethylation levels in different tissues. Mouse brain (MB) was used as a positive control and Drosophila DNA a negative control.

$F_{6,20} = 3.041, p = 0.027$; Position 3: $F_{6,20} = 1.598, p = 0.199$; Position 4: $F_{6,20} = 2.641, p = 0.047$). By contrast, developmental time did not had an effect on methylation percentage in any of the positions (Postion 1: $F_{1,20} = 0.101, p = 0.753$; Position 2: $F_{1,20} = 3.271, p = 0.085$; Position 3: $F_{1,20} = 1.060, p = 0.315$; Position 4: $F_{1,20} = 1.914, p = 0.181$).

Response of global methylation patterns to thermal stress

We scanned 67 individuals, and from the three primer combinations we obtained a total of 719 loci, of which 677 were susceptible to methylation (MSL) and 354 were polymorphic (52% of the total MSL). Of the total number of loci, 42 were not susceptible to methylation (NML) and 35 were polymorphic (83% of the total NSL). The perMANOVA results showed that developmental temperature ($F_{3,57} = 3.39, p < 0.001$) and family ($F_{1,57} = 1.45, p < 0.05$) had a significant effect on methylation patterns, however the interaction between these two predictors was not significant ($F_{14,43} = 0.91, p > 0.05$). Using a pairwise perMANOVA we analyzed the effect of temperature, controlling for family. The results reveal that methylation patterns of individuals developing at 28°C were significantly different from those in any of the other temperature (26°C vs 28°C $F_{1,36} = 3.20, p < 0.05$, 28°C vs 30°C $F_{1,35} = 4.68, p = 0.001$, 28°C vs 32°C, $F_{1,28} = 8.15, p = 0.001$). The methylation patterns of the individuals at 26°C and 32°C grouped significantly different from each other ($F_{1,28} = 2.78, p < 0.01$) but neither of these temperatures were different to those at 30°C (26°C vs 30°C $F_{1,35} = 1.27, p > 0.05$, 30°vs 32°C $F_{1,27} = 1.49, p > 0.05$). The differences between temperature conditions are given in table S2. We also ran a pairwise perMANOVA to test for differences between families, controlling for temperature. The results indicate that just

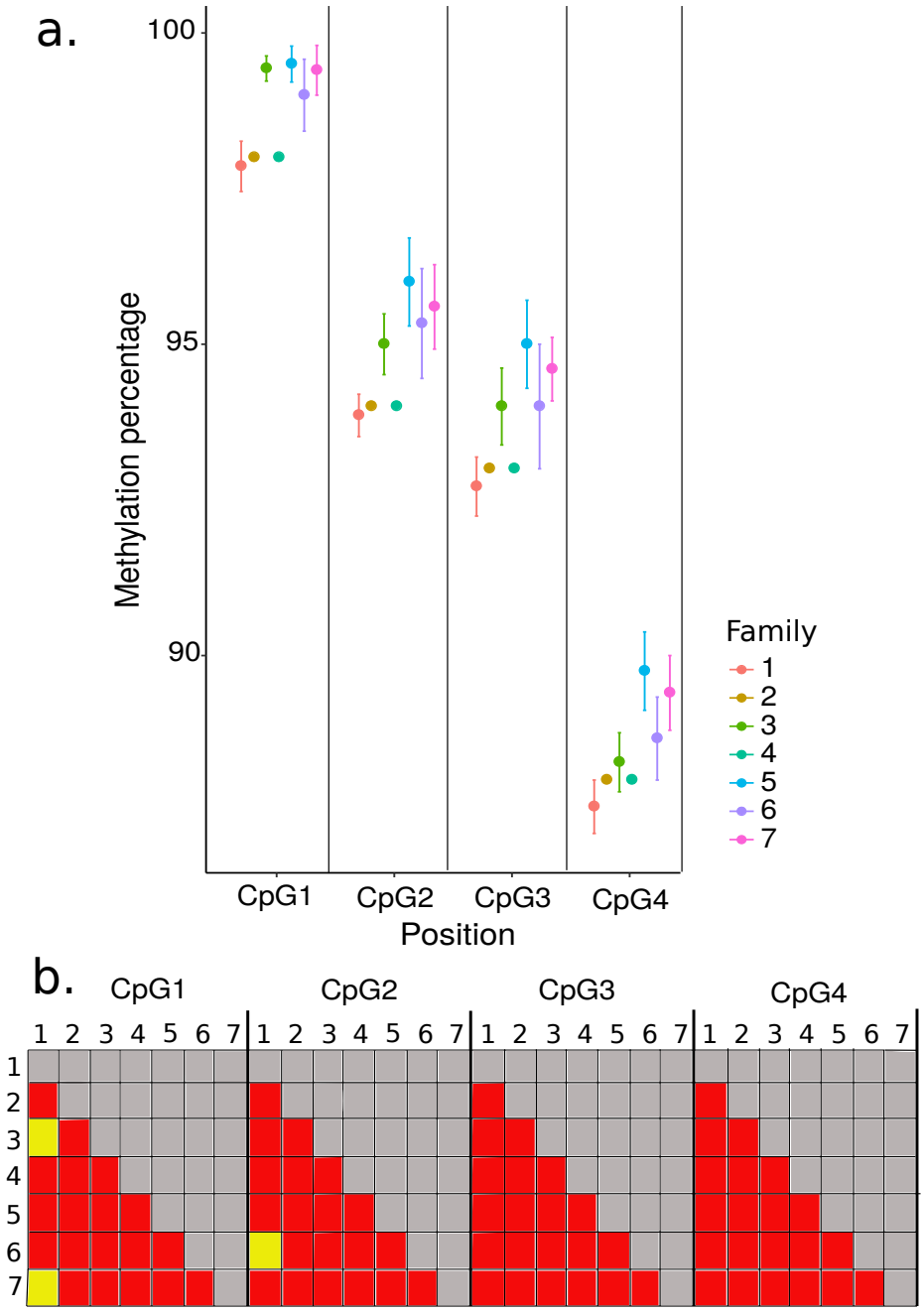

Figure 2. Effect of family on the four Hsp70 CpGs analyzed. Shown is the raw data with error bars representing the standard error. **A**. Methylation percentage of the four CpGs analyzed in Hsp70 color coded by family. **B**. Pairwise post hoc results of each position analyzed in Hsp70, yellow boxes are significant p values, red boxes are non-significant values.

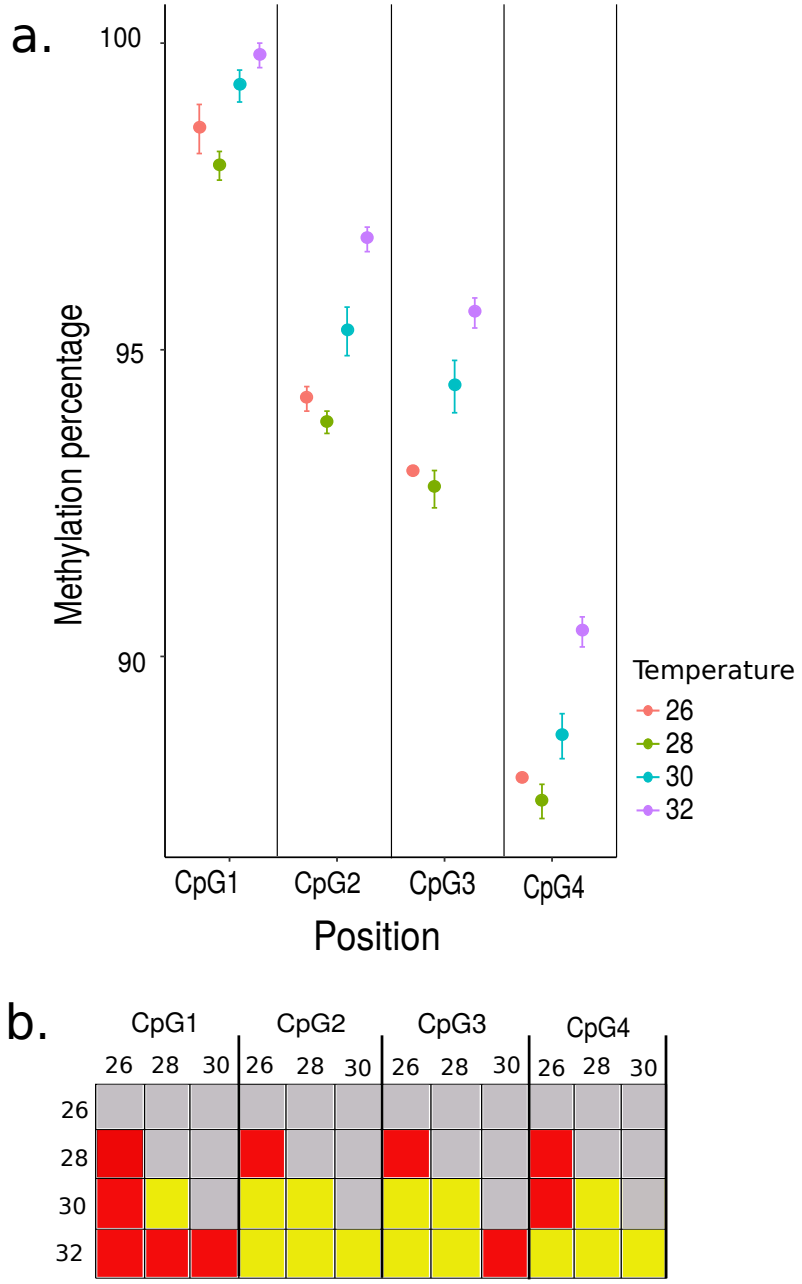

Figure 3. Effect of temperature on the four Hsp70 CpGs analyzed. Shown are the raw data with error bars representing the standard error. **A**. Methylation percentage of the four anlyzed postion in the Hsp70 color coded by temperature treatment. **B**. Pairwise post hoc results of each position analyzed in the Hsp70, yellow boxes are significant p values, red boxes are non-significant values.

Table 1. Effect of temperature on methylation pattern variation, F1 individuals. Shown are the p values from 1,000,000 random permutations of the individual's euclidean distance to the centroid of the PCoA. Pairwise comparison was done between the four temperature treatments to which the first generation was exposed to.

	26°C	28°C	30°C
26°C			
28°C	< 0		
30°C	> 0.05	< 0	
32°C	> 0.05	< 0	> 0.05

a few families differ between each other in their methylation patterns being family five and six the ones that differ from several families. Family six significantly differs from family one and five (1 vs 6 $F_{1,16} = 2.83, p > 0.05$, 6 vs 5 $F_{1,16} = 3.71, p < 0.01$). While family 5 significantly differed from family two, three and six (5 vs 2 $F_{1,17} = 3.32, p < 0.05$, 5 vs 3 $F_{1,24} = 2.02, p > 0.05$). The pairwise comparison are presented in table S4.

Methylation variation. We evaluated the variability of the methylation patterns within each temperature condition. We found in the first generation, that individuals at 28°C have less variability in their methylation patterns. The level of dispersion of this group is significantly different from the individuals at 26°C, 32°C, and 30°C (*p-values* are given in table 1).

Not susceptible methylation loci. The not susceptible methylation loci (NML) are those that were not methylated in any of the samples. Because the loci are not susceptible to methylation, the presence or absence of these loci represent genetic mutations. Therefore, these loci are useful to evaluate genetic variation across the samples. In the first generation, we found 42 NML, on which temperature had a significant effect ($F_{3,57} = 1.41, p < 0.05$; Fig. 4 **B**) but family did not have an effect ($F_{6,57} = 1.18, p > 0.05$; Fig. 4 **B**).

However, only the individuals from temperature 28°C vs the individuals at 32°C ($F_{1,28} = 1.96, p < 0.01$; Table S3 are significantly different).

Discussion

The results of our first set of experiments confirm an average of 9% of global DNA methylation in all cockroach tissues investigated. By contrast, hydroxymethylation was present in the cockroach brain only, with similar levels to those reported in the mouse brain. Our results further show that methylation is highly sensitive to thermal stress. We found that methylation at all Hsp70 cytosines was sensitive to temperature. However, genotype effects on methylation were detected only at some sites. Finally, we found that global methylation profiles were affected by both temperature and the genotype showing that methylation variation is much lower at 28°C than at other temperatures.

Global methylation

Our first aim was to identify and quantify DNA methylation and hydroxymethylation at tissue specific level in *Diploptera punctata*. We found overall high levels of methylation in all tissues. Our results are concordant with previous work that have reported DNA 5-mC levels between 2% and 14% in Blattodea [8, 26]. Hydroxymethylation, by contrast, was only found in the head supporting the hypothesis that tissue-specific DNA 5-hmC might be implicated in neural development and neural plasticity [45, 10]. Hydroxymethylation is an epigenetic mark poorly explored in insects, as it has only been studied in the honeybee. The presence of hydroxymethylation has been linked to neural tissues in mammals and its presence in

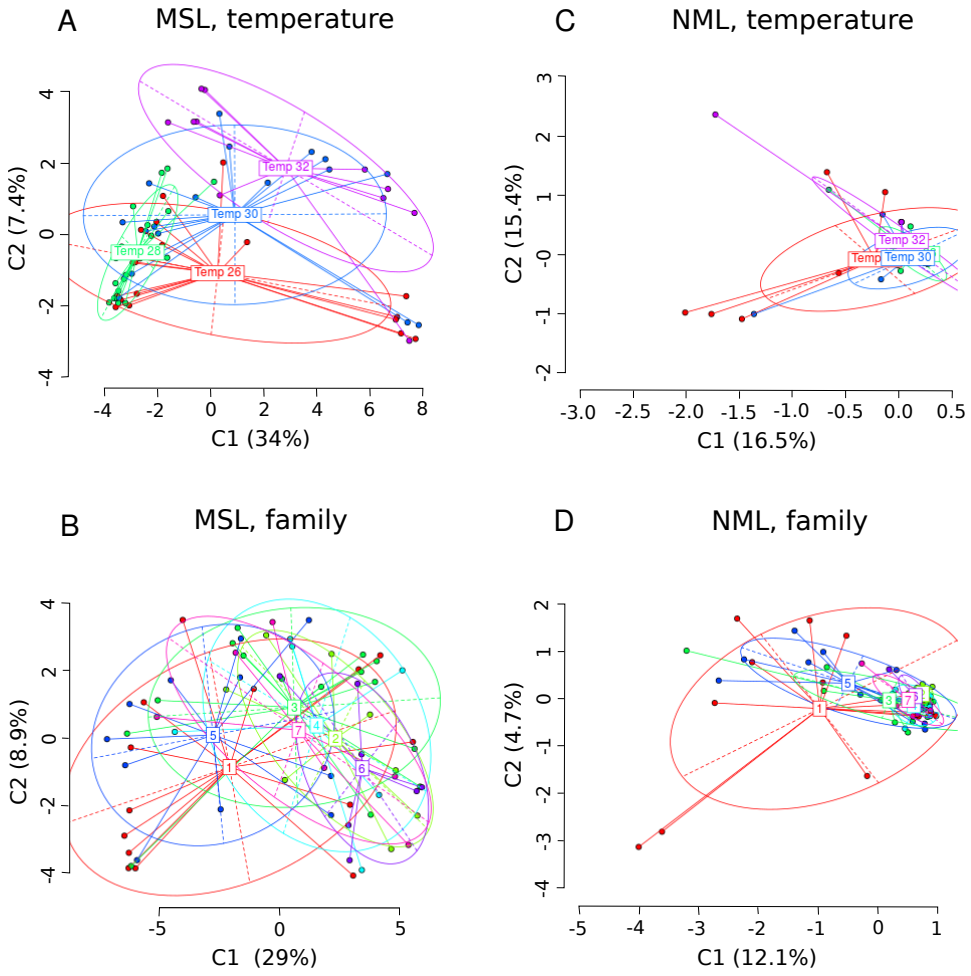

Figure 4. Principal coordinate analysis. The figure shows the principal coordinate analysis for epigenetic (methylation sensitive loci; MSL) and genetic (not methylation sensitive loci; NSL). The two coordinates presented are shown with the percentage of variation explained by them. The points represent the individuals and the group labels the centroid for the individuals in each group. The ellipses represent the mean dispersion of the points around the centroid. The individuals are grouped by temperature (**A** and **C**) and family (**B** and *D*)

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the brain suggests a link to flexible alterations in the chromatin. It has been hypothesized that neurons need to have a flexible epigenetic mechanism because they cannot divide. Therefore if an epigenetic mark, such as methylation, need to be rearranged (due to, for example, cellular requirements or environmental stressors) neurons need to rely on a demethylation process that does not require cell duplication [45]. The fact that hydroxymethylation is only present in the brain is especially interesting due to the complex neural and behavioural structure of cockroaches. In fact, it has recently been shown that cockroaches hold the largest chemosensory gene repository known in arthropods [42]. *Blattella germanica* has the largest family of odorant binding proteins and ionotropic receptor proteins, and the second largest number of gustatory proteins. The large chemosensory repository present in the cockroach suggests that these proteins may play an important role in the chemical ecology of the species, for example in sex and aggregation pheromones or the remarkable evolution of sugar aversive strains [42]. Further studies on the sites where hydroxymethylation is enriched in the brain of the cockroach will be necessary to elucidate whether hydroxymethylation is related in any way with the chemosensory repository.

Methylation level in Hsp70

Our second research aim was to investigate whether DNA methylation in the intragenic region of the gene Hsp70 was susceptible to thermal stress across seven different genotypes. In this regard, we found that temperature and the genotype had an effect on methylation percentage. In several species, it has been observed that thermal stress causes upregulation of Hsp70. The level of upregulated expression is often correlated with thermal

stress resistance [27]. For example, a study performed by Hu et al [27] investigated if the divergence in thermal plasticity of two invasive congenic fruit fly species (*Bactrocera correcta* and *Bactrocera dorsalis*) is associated with Hsp expression levels. *B. dorsalis* is a widely distributed species, while *B. correcta* is narrowly distributed. They found evidence suggesting that Hsp70 may be involved in regulating thermal plasticity, as the more widespread species had greater ability to express Hsp70 [27]. Other studies in invertebrates have corroborated the relation between thermal plasticity and thermal resistance to Hsp gene expression [46, 24, 15, 4]. However, the molecular mechanism that regulates Hsp70 expression is poorly studied in invertebrates. A study performed on the mollusc *Biomphalaria glabratahe* found that methylation of the Hsp70 responded to heat shock [29], proposing methylation as an important regulatory mechanism of Hsp70 in invertebrates. In insects, methylation is enriched in the gene body and it is linked to gene activation [16, 21]. We found higher levels of methylation at the highest temperature (32°C), which could mean high rates of gene expression. This needs to be confirmed in future work looking at the relation between methylation and gene expression in this specific case. It is also crucial to understand the physiological and biological implications of the observed methylation percentage and investigate if it has any effect on, for example, gene expression, alternative splicing or the phenotype.

Global methylation profiles

Methylation variation Our third and final aim was to investigate how global DNA methylation profiles respond to thermal stress. We used MS-AFLPs to scan for epigenetic profiles across the genome and found that the environment and the genotype affected methylation patterns. Methylation

patterns were more similar in organisms that developed at the same temperature. Furthermore, we observed an interesting pattern in the variation of methylation profiles in each treatment. Methylation patterns vary more stochastically in all temperature regimes, except for the 28°C treatment, in which all the samples clustered together. A possible explanation for this might be that 28°C is the closest to the optimal developmental temperature. The results from several studies propose that when organisms are exposed to stressful conditions methylation patterns vary stochastically. Several studies have found a link between DNA methylation and environmental stress, describing higher variability in DNA methylation when organisms are under environmental stress. If the same methylome or phenotype is expressed constantly over generations through transitory methylation patterns, then these patterns are expected to become common and fixed in the population, and therefore may contribute to epigenetic differentiation between populations. Therefore, DNA methylation that is stress or environmentally induced might influence an individual or the population fitness about local environments. Controlled experiments show that several environmental stressors such as low nutrients, salinity, or pathogen attacks can induce methylation variation [28, 39, 50, 33]. This variability has been recorded in natural and lab conditions. For example, Leung et al described that in *Chrosomus neogaeus*, the fine-scale dace, under unpredictable environments, stochastic epigenetic variation is induced. However, they reported that this variation will be highly influenced by the genotype [31]. In several experiments on stress-related methylation, it has been described that the stability of these marks is highly variable. The marks have been recorded to be stable from several hours up to several generations [28]. Other studies report that stochastic DNA methylation variation occurs just several hours

following the exposure to the stressful environment [36]. For instance, a study on three species of coral demonstrated that DNA methylation variation influences their tolerance to thermal stress and ocean acidification [13].

Following our results, for future research, we propose to study the costs associated with high rates of stochastic epimutations and establish for how long these patterns are stable. A wider study focusing on species that differ in their life history would produce interesting findings on the cost of generating epigenetic stochasticity under stressful environments.

Our results also demonstrate that epigenetic variation is greater than genetic variation. This has been widely reported in the literature where especially in natural population epigenetic loci are more variable than genetic loci [18, 28, 36]. This supports the idea that epigenetic variation can help organisms to cope with environmental changes more rapidly than genetic variation. Indeed, in several invasive species, which are characterized by low genetic variation, methylation variation is higher than genetic variation [25]. This has also been observed in populations with naturally low levels of genetic variation (e.g. clonal species), in which increased epigenetic diversity may help overcome the naturally low amount of genetic variation. We were expecting to find low genetic diversity, as the cockroaches have been kept in the laboratory for over a decade, and indeed we find low genetic variation between families. However, we note that we evaluated genetic variation based on a relatively low number of NML. To further corroborate this finding more exhaustive studies of genetic variation need to be conducted using e.g. AFLPs.

Our study failed to find a correlation between genetic and epigenetic variation. This means that specific genetic profiles are not correlated to

specific methylation patterns. However, the lack of correlation between the genetic and epigenetic matrices and the fact that a large amount of epigenetic variation could be explained by the environment, suggests that several epigenetic marks might be independent of the genome in*D. punctata*. It would then be necessary to examine which methylated regions are correlated with genotype, and which are correlated to the environment to gain an understanding of the function of methylation marks associated with the genotype as opposed to the environment.

The genetic dependency of epigenetic variation is not well described, but it is possible to be species or taxa dependent [34]. It is important to bear in mind that the fact that the environment determines a high amount of epigenetic patterns, does not mean that these patterns have a functional link or that these are under selection. To address the functionality of methylation in response to temperature in *D. punctata* we would need to explore in more detail wherein the genome methylation changes are occurring.

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Disclosure statement

The authors declare that they have no competing interests.

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Supplementary material

Table 2. Effect of temperature on methylation patterns, F1 individuals. Shown are the p values of the pair-wise perMANOVA analysis. The pairwise comparison was made between the four different temperature regimes to which the first generation individuals were exposed to. The significant p values are coloured in yellow and marginally significant values are coloured in orange.

	26°C	28°C	30°C
26°C			
28°C	< 0.05		
30°C	> 0.05	0.001	
32°C	< 0.01	0.001	0.09

Table 3. Effect of temperature on NML, F1 individuals. Shown are the p values of the pair-wise perMANOVA analysis. The pairwise comparison was made between the four different temperature regimes to which the first generation individuals were exposed to. The significant p values are coloured in yellow.

	26°C	28°C	32°C
26°C			
28°C	> 0.05		
30°C	> 0.05	> 0.05	
32°C	> 0.05	< 0.01	> 0.05

Table 4. Effect of family on methylation patterns Shown are the *p* values from a pairwise perMANOVA. In yellow are the significant values, in orange the marginal significant effects.

First generation

	1	**2**	**3**	**4**	**5**	**6**
1						
2	0.055					
3	0.058	0.6				
4	0.19	0.92	0.76			
5	0.25	0.03	0.05	0.08		
6	0.02	0.09	0.22	0.19	0.005	
7	0.36	0.4	0.72	0.35	0.3	0.24

Table 5. List of primers and adapters. Sequences of primers and adapters used in for ligation pre-selective PCR and selective PCR. The sequences of primers and adapters were taken from Amarasinghe et al 2014 [1]. The primer combination used for the selective PCR was the primer forward A (FA) with the primer reverse A (RA), the primer foward B (FB) with the primer reverse B (RB), and the primer foward C (FC) with the primer reverse C (RC).

Adapter/ primer name	**Sequence (5'-3')**
Ligation	
EcoRI- F	CTCGTAGACTGCGTACC
EcoRI-R	AATTGGTACGCAGTCTAC
HpaII-MspI–F	GACGATGAGTCTAGAA
HpaII-MspI–R	CGTTCTAGACTCATC
Pre-selective PCR	
EcoRIpre (EcoRI + 0)	GACTGCGTACCAATTC
HpaII-MspI pre (HpaII-MspI + A)	GATGAGTCTAGAACGGA
Selective PCR	
Eco-AG (6-FAM)(FA)	GACTGCGTACCAATTCAG
Eco-AC (6-HEX)(FB)	GACTGCGTACCAATTCAC
Eco-AT (6-AT)(FC)	GACTGCGTACCAATTCAT
HpaII-MspI-ACT(RA)	GATGAGTCTAGAACGGACT
HpaII-MspI-AAT(RB)	GATGAGTCTAGAACGGAAT
HpaII-MspI-ATT(RC)	GATGAGTCTAGAACGGATT