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1 **DNA methylation patterns respond to**
2 **thermal stress in the viviparous**
3 **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

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

44 Among the main epigenetic processes, DNA methylation is the best
45 studied epigenetic mark, which involves the addition of a methyl group on
46 the fifth position of the cytosine. In mammals, DNA methylation is mainly
47 enriched in regulatory regions and is associated with gene silencing [8, 16].
48 By contrast, in invertebrates DNA methylation is enriched in exons and is
49 associated with gene activation . DNA methylation is highly dynamic as it
50 can vary in response to, for example, environmental factors, requirements
51 of the cell, or developmental stage [22]. DNA hydroxymethylation (DNA
52 5-hmC), by contrast, is a largely unexplored epigenetic mechanism that
53 is presumably involved in gene upregulation and active demethylation
54 processes [35]. DNA hydroxymethylation is mainly found in the nervous
55 system, suggesting important and specific neural and developmental func-
56 tionality [45, 40].

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

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

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

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

89 In this paper, we present the first investigation of DNA methylation
90 and hydroxymethylation in the cockroach *Diploptera punctata* and its
91 response to thermal stress. *Diploptera punctata* is among the very few
92 truly viviparous insects and the only truly viviparous cockroach. It belongs
93 to Blattodea, a group that presents several adaptations to thermal stress
94 [7]. Our first research aim was to investigate if DNA methylation and
95 hydroxymethylation are present in *Diploptera punctata*. To achieve this, we

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

107 **Methods**

108 **0.1 Animal maintenance**

109 *D. punctata* colonies had been established for over 10 years in the laboratory
110 and were maintained in plastic containers (30 × 22 × 20 cm) at 25°C on a
111 12:12h light: dark cycle and fed with blended dog food (WAGG Complete
112 Dog Food) and water provided ad libitum.

113 **0.2 Global DNA Methylation percentage**

114 **Dissections** Cockroaches were dissected in bath saline solution (135 Mm
115 NaCl, 5 mM KCl, 4mM MgCl₂ 6H₂O, 2mM CaCl₂ 2H₂O, 5 mM TES, 36
116 mM sucrose). We dissected the legs, head, fat body and embryos. Embryos
117 were obtained between days 45 and 55 of pregnancy (59-75% of total de-
118 velopment [47]). At this stage, the embryos are between 4 and 5 mm long.

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

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

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

141 **Temperature manipulation**

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

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

163 **Response of Hsp70 gene methylation to thermal stress**

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

166 Hsp70 available sequences from closely related species. In total we col-
167 lected three sequences from three species: *Blatella germanica* (Accession
168 No: PYGN01000002.1:4236897-4238334), *Periplaneta americana* (Accession
169 No: KY661334.1) and *Cryptocercus punctulatus* (Accession No: JQ686949.1).
170 The sequences were aligned on Clustal Omega [11]. We then used PriFi
171 [20] (<https://services.birc.au.dk/prifi/main.py>) to design multiple
172 set of primers. This tool is useful for designing primers from multiple
173 sequence alignments derived from phylogenetically related species, in
174 particular when working with organisms without a reference genome.
175 Two different parameters were entered into the system for Hsp70 align-
176 ment. The sequence of the primers that amplified successfully the de-
177 sired fragment is: Fw 5'-AAGGGTCATGGAGAACGCAA-3' and Rv 5'-
178 CTCTTCATGTTGAAGCAGTA-3'. For the PCR amplification, we added
179 2µl (150ng/µl) of DNA to the PCR mix (Foward primer 1µl (0.4µM), Re-
180 verse primer 1µl (0.4µM), PCR mix 12.5µl, Nuclease free water 8.5µl) with
181 the following PCR conditions: 95 °C for 4 min, followed by 35 cycles of 95
182 °C for 15 sec, 61 °C for 1 min, 72 °C for 1.15 min and 72 °C for 10 min.

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

190 **Bisulfite conversion and pyrosequencing** DNA was bisulphite con-
191 verted using the PyroMark PCR Kit (Qiagen) with 200ng. We aligned

192 *Diptera punctata* Hsp70 sequence with the Hsp70 sequence of *B. glabratahe*.
193 In the conserved regions we identified, and selected for further anal-
194 ysis, a region with four CpGs that was found to be methylated in *B.*
195 *glabratahe* [29]. We used PyroMark Assay Design software (Qiagen)
196 to design a set of a forward, reverse and sequencing primers, which
197 are as follow: Fw 5'-ATTTAAGTTTAAGAAGGTGAGAGAGTAATG-
198 3', Rv 5'-CTCCTTTCCTATTAATTTTTCAACTACTA -3', sequence 5'-
199 GGTGTTTTATAAATTGAGGTTATT-3'. The reverse primer is biotinylated
200 at 5' end. The PyroMark Q24 (Qiagen) was used for pyrosequencing using
201 PyroMark Q24 Advanced Reagents kit (Qiagen). From the PCR sample 10µl
202 was used for each pyrosequencing reaction using the sequencing primer
203 5'- TTGTTGGTGGTAGTTTT-3' and were performed in duplicate. Pyro-
204 Mark PCR Kit (Qiagen) was used to carry out methylation-specific PCRs as
205 specified by the manufacturer.

206 **Statistical methods** All four positions were analyzed independently.
207 Data were analysed using linear mixed-effects models in the R environment
208 [41], using the packages lme4 and car [19, 6]. The percentage of methylation
209 at each cytosine was used as a response variable, the genotype (six levels),
210 temperature (four levels) and developmental time were considered as a
211 fixed factor.

212 **Response of global methylation patterns to thermal stress**

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

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

225 For MS-AFLPS a total of 67 organisms were screened. All samples were
226 processed in duplicate. We followed the protocol in Amarasinghe et al
227 (2014) [1], with some modifications (see primers in table S5). We digested
228 DNA in two separate reactions. The first one used EcoRI (0.05µl NEB, 20
229 000 units/ml) + MspI (0.025µl NEB, 20 000 units/ml). The second one used
230 EcoRI + HpaII (0.5µl NEB, 20 000 units/ml). We added 5µl of DNA to
231 the two independent digestion mixtures (EcoRI, MspI/HpaII, 1µl NEB cut
232 buffer 10X, and 3µl of ddH₂O). The reaction was incubated for three hours
233 at 37 °C. Immediately after digestion, we added 5µl of the digested product
234 to the ligation reaction (0.25µl T4 DNA ligase NEB (400000 units/ml), 1 µl
235 of NEB ligase buffer, 1µl of EcoRI adapter (5 pmol), 1µl HpaII-MspI (50
236 pmol), and 1.75 ul of ddH₂O). The ligation reaction was incubated at 37
237 °C for three hours and left overnight at room temperature. Then, we ran
238 a pre-selective PCR (pPCR) by adding 5µl of the ligated product to the
239 pPCR mix (1.25 µl pre-selective EcoRI primers (0.5 µM), 1.25µl pre-selective
240 HpaII-MspI primers (0.5 µM), 12.5 µl of PCR master mix Agilent, Paq5000
241 Hotstart PCR Master Mix) and the following PCR conditions: 94 °C for 2
242 min, followed by 35 cycles of 94 °C for 30 sec, 58 °C for 1 min, 72 °C for
243 1 min and 72 °C for 5 min. Then we ran selective PCR (sPCR) in which

244 three different primers were used. We used 5µl of the pPCR product as
245 a DNA template, which was added to the following mix (0.5 µl selective
246 EcoRI primer (0.5µM), 0.5µl of selective HpaII/MspI primers (0.5µM) and
247 5µl of PCR master mix (Agilent, Paq5000 Hotstart PCR Master Mix), with
248 the following PCR conditions: 94 °C for 2 min, followed by 13 cycles of
249 94 °C for 30 sec, 65 °C (decreasing 0.7 °C per cycle) for 30 sec, followed by
250 30 cycles of 94 °C for 30 sec, 56 °C for 30 sec, 72 °C for 1 min, and a final
251 extension of 72 °C for 5 min. Each of the forward selective primers was
252 marked with a different fluorophore (6-FAM, HEX, ROX). The fluorophore
253 allows the identification of the fragment after capillary electrophoresis.
254 Finally, we mixed 0.5µl of the sPCR product with 0.4µl of 500 LIZ dye Size
255 Standard (ThermoFisher) and 9µl of Hi-Di formamide (ThermoFisher). The
256 samples were then sent to the University of Manchester sequencing facility
257 for fragment analysis.

258 **Statistical analysis**

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

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

272 **Methylation variation** To determine whether the variation in methyla-
273 tion patterns is influenced by temperature we obtained the distance of each
274 coordinate from the PCoA to the center of each respective group using the
275 Euclidean distance formula:

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

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

278 To determine whether the observed differences in methylation distance
279 between temperature groups were greater than we would expect to see
280 by chance alone, and therefore statistically significant, we ran a series of
281 pairwise comparisons of the different developmental temperatures (e.g.
282 26°C vs 28°C, 26°C vs 30°C, etc) and compared them to a null distribution
283 of differences obtained in 10^6 randomly generated permutations of the data.
284 We created permutations by reassigning the observed data points between
285 the temperature groups, subject to the constraint that the number of ob-
286 servations could not change within temperature groups. This constraint is
287 important because there are different numbers of observations in the differ-
288 ent temperature groups, and the variance of methylation per temperature
289 group depends on the number of observations. For each comparison, the
290 *p-value* is the proportion of the null distribution in which the difference
291 between temperatures was as great as, or greater than, in the observed
292 data.

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

303 **Genetic and epigenetic correlation** To analyze the correlation between
304 epigenetic variation and genetic variation we performed a Mantel test using
305 the R *vegan* package [37]. The Mantel test is a correlation between entries
306 of two dissimilarity matrices. To run the Mantel test we first created two
307 different distance matrices using the Euclidean method. We created the
308 first matrix using the MSL and another using NML for both generations
309 separately. For the Mantel test, we used the Pearson method and ran
310 1,000,000 permutations.

311 **Results**

312 **Global methylation levels**

313 We found evidence of DNA methylation in all tissues (average DNA
314 methylation level 8.8%), with no significant difference among them (GLM
315 $F_{3,25} = 0.9535, p > 0.05$). We used adult *Drosophila melanogaster* as a neg-
316 ative control, (no DNA methylation), and, as expected we did not find

317 evidence of methylation in *Drosophila melanogaster*. Further, all *Diploptera*
318 *punctata* tissues showed significantly higher levels than *Drosophila* (HSD,
319 *Drosophila* vs embryo, $t - ratio = -9.81, p < 0.0001$, *Drosophila* vs fat
320 body, $t - ratio = -7.71, p < 0.0001$, *Drosophila* vs head, $t - ratio =$
321 $-9.71, p < 0.001$, *Drosophila* vs leg, $t - ratio = -9.75, p < 0.001$;Fig.
322 1 A). In contrast to DNA methylation, global DNA hydroxymethylation
323 was found only in the cockroach head. We found that 0.75% of the cy-
324 tosines were hydroxymethylated (Fig. 1 B). For the hydroxymethylation
325 assay we also used adult individuals of *Drosophila melanogaster* as a neg-
326 ative control, and mouse brain as a positive control. Interestingly, we
327 found similar levels of 5-hmCs in the head of *D. punctata* as those found in
328 the mouse brain (0.22%). The levels of hydroxymethylation in the mouse
329 brain and in the cockroach head were not significantly different from each
330 other ($z - value = -1.51, p > 0.05$). The hydroxymethylation levels of
331 *Drosophila* were not different from the levels of the embryo, fat body and
332 leg (*Drosophila* vs embryo $z - value = 0.123, p > 0.05$ *Drosophila* vs fat body
333 $zvalue = 0.79, p > 0.05$, *Drosophila* vs leg $z - value = 1.29, p > 0.05$).

334 **Response of Hsp70 gene methylation to thermal stress**

335 We analyzed four cytosines in the intergenic region of the Hsp70. The four
336 positions analyzed were highly methylated (98.806%, 94.838%, 93.774% and
337 88.483% in the first, second, third and fourth positions respectively). All
338 four CpGs analyzed showed significant temperature effects on methylation
339 (Position 1: $F_{3,20} = 4.575, p = 0.013$; Position 2: $F_{3,20} = 12.50, p = 7.91e -$
340 05 ; Position 3: $F_{3,20} = 5.986, p = 0.004$; Position 4: $F_{3,20} = 9.048, p =$
341 0.0005). Family had an effect on methylation percentage at three of the
342 four positions analyzed (Position 1: $F_{6,20} = 3.922, p = 0.009$; Position 2:

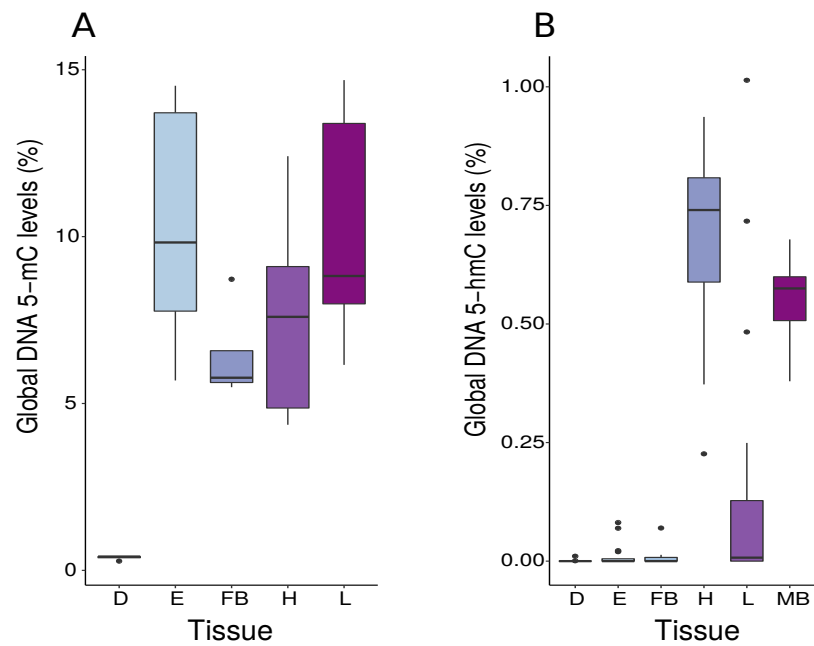


Figure 1. Methylation and hydroxymethylation levels in *Diploptera punctata*. **A.** Global methylation levels from four different tissues (embryo (E), fat body (FB), head (H), and legs (L)). *Drosophila* DNA (D) was used as a negative control (no DNA methylation). **B.** Global DNA hydroxymethylation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

343 $F_{6,20} = 3.041, p = 0.027$; Position 3: $F_{6,20} = 1.598, p = 0.199$; Position 4:
344 $F_{6,20} = 2.641, p = 0.047$). By contrast, developmental time did not had
345 an effect on methylation percentage in any of the positions (Position 1:
346 $F_{1,20} = 0.101, p = 0.753$; Position 2: $F_{1,20} = 3.271, p = 0.085$; Position 3:
347 $F_{1,20} = 1.060, p = 0.315$; Position 4: $F_{1,20} = 1.914, p = 0.181$).

348 **Response of global methylation patterns to thermal stress**

349 We scanned 67 individuals, and from the three primer combinations we
350 obtained a total of 719 loci, of which 677 were susceptible to methyla-
351 tion (MSL) and 354 were polymorphic (52% of the total MSL). Of the
352 total number of loci, 42 were not susceptible to methylation (NML) and
353 35 were polymorphic (83% of the total NSL). The perMANOVA results
354 showed that developmental temperature ($F_{3,57} = 3.39, p < 0.001$) and
355 family ($F_{1,57} = 1.45, p < 0.05$) had a significant effect on methylation
356 patterns, however the interaction between these two predictors was not
357 significant ($F_{14,43} = 0.91, p > 0.05$). Using a pairwise perMANOVA we
358 analyzed the effect of temperature, controlling for family. The results re-
359 veal that methylation patterns of individuals developing at 28°C were
360 significantly different from those in any of the other temperature (26°C
361 vs 28°C $F_{1,36} = 3.20, p < 0.05$, 28°C vs 30°C $F_{1,35} = 4.68, p = 0.001$,
362 28°C vs 32°C, $F_{1,28} = 8.15, p = 0.001$). The methylation patterns of the
363 individuals at 26°C and 32°C grouped significantly different from each
364 other ($F_{1,28} = 2.78, p < 0.01$) but neither of these temperatures were dif-
365 ferent to those at 30°C (26°C vs 30°C $F_{1,35} = 1.27, p > 0.05$, 30°vs 32°C
366 $F_{1,27} = 1.49, p > 0.05$). The differences between temperature conditions are
367 given in table S2. We also ran a pairwise perMANOVA to test for differences
368 between families, controlling for temperature. The results indicate that just

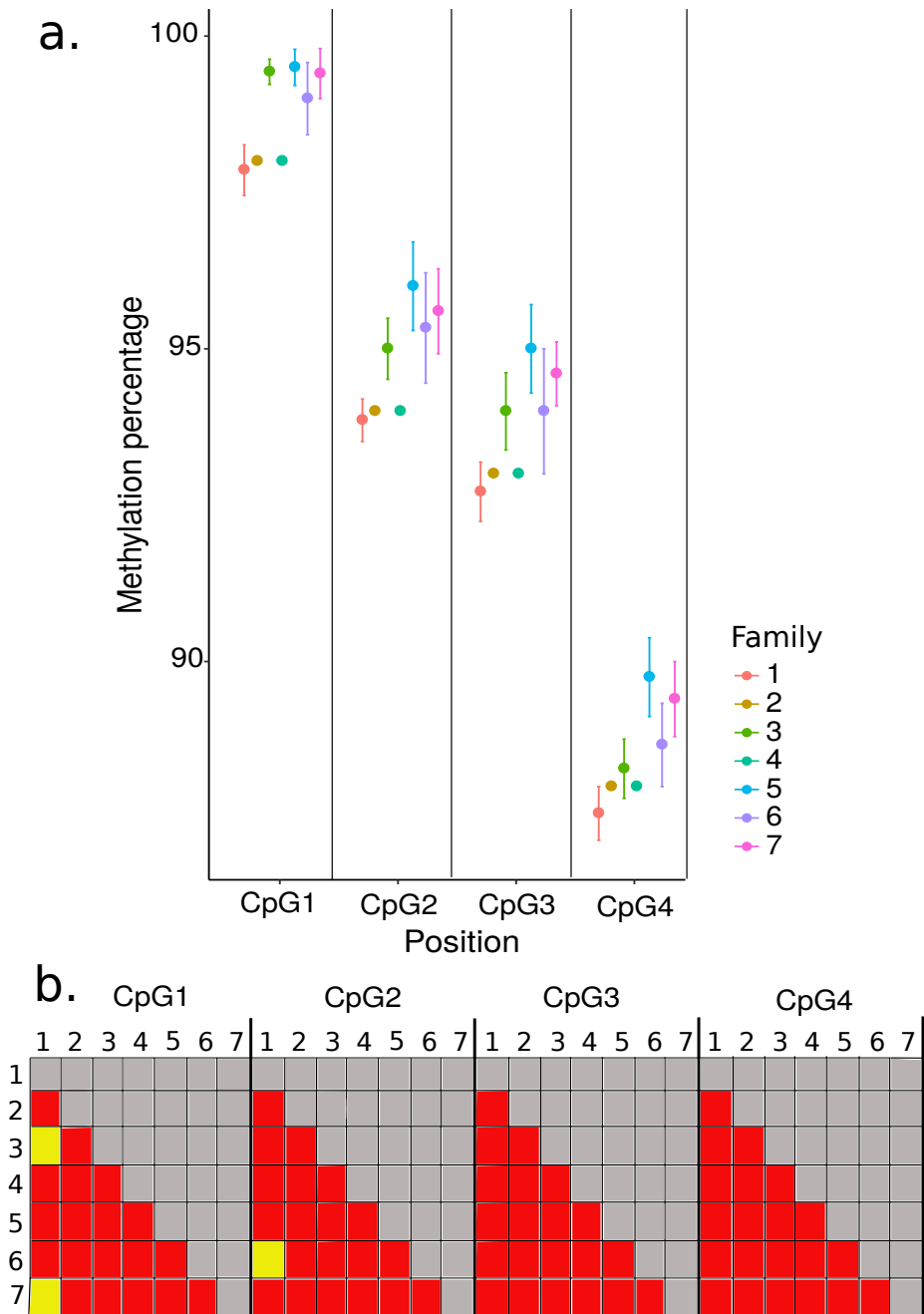


Figure 2. Effect of family on the four Hsp70 CpGs analyzed. Shown is the raw data with error bars representing the standard error. **A.** Methylation percentage of the four CpGs analyzed in Hsp70 color coded by family. **B.** Pairwise post hoc results of each position analyzed in Hsp70, yellow boxes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

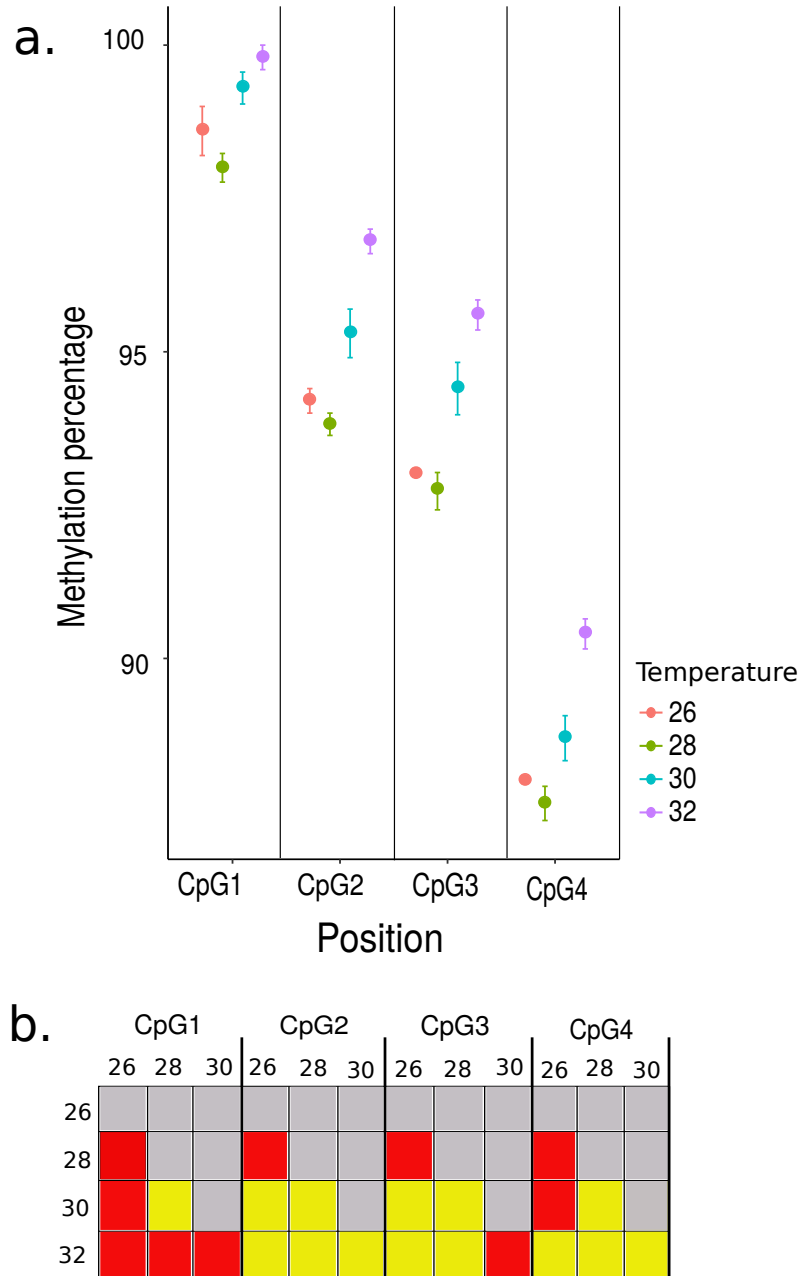


Figure 3. Effect of temperature on the four Hsp70 CpGs analyzed. Shown are the raw data with error bars representing the standard error. **A.** Methylation percentage of the four analyzed position in the Hsp70 color coded by temperature treatment. **B.** Pairwise post hoc results of each position analyzed 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

369 a few families differ between each other in their methylation patterns being
 370 family five and six the ones that differ from several families. Family six sig-
 371 nificantly differs from family one and five (1 vs 6 $F_{1,16} = 2.83, p > 0.05$, 6 vs
 372 5 $F_{1,16} = 3.71, p < 0.01$). While family 5 significantly differed from family
 373 two, three and six (5 vs 2 $F_{1,17} = 3.32, p < 0.05$, 5 vs 3 $F_{1,24} = 2.02, p > 0.05$).
 374 The pairwise comparison are presented in table S4.

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

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

387 However, only the individuals from temperature 28°C vs the individuals at
388 32°C ($F_{1,28} = 1.96, p < 0.01$; Table S3 are significantly different).

389 **Discussion**

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

400 **Global methylation**

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

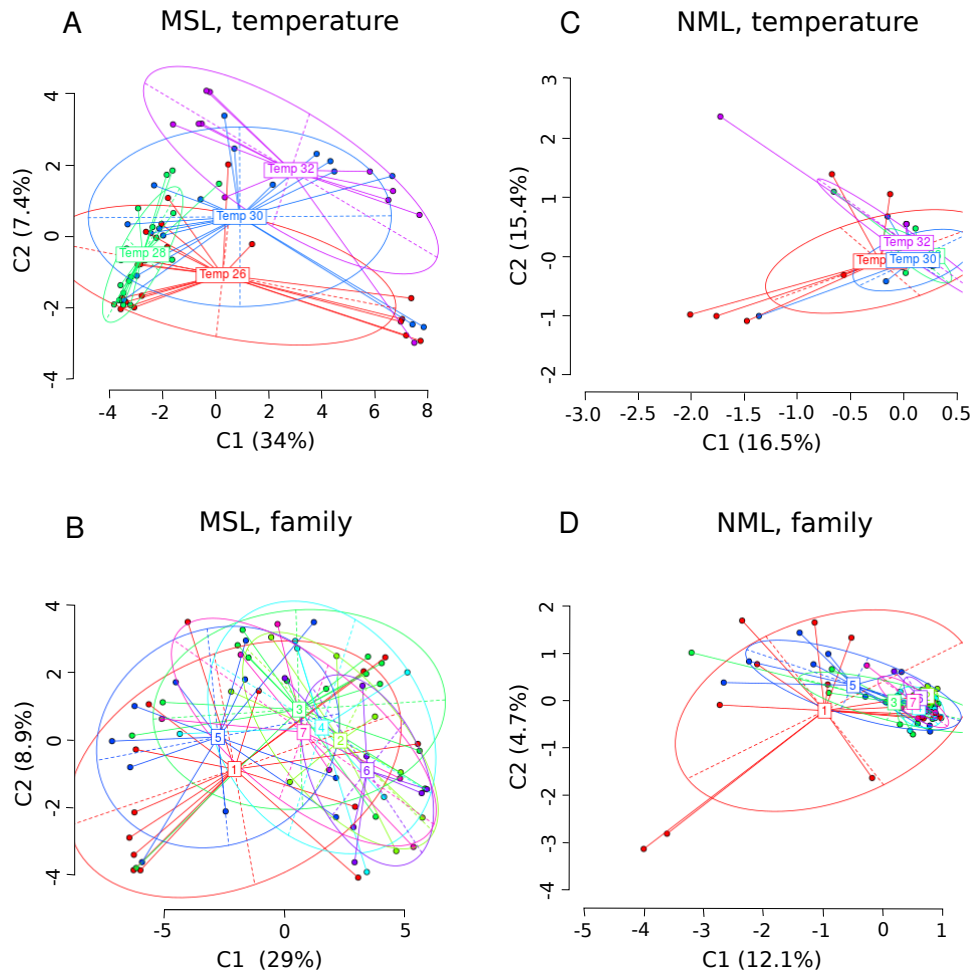


Figure 4. Principal coordinate analysis. The figure shows the principal coordinate analysis for epigenetic (methylation sensitive loci; MSL) and genetic (not methylation sensitive loci; NSL). The two coordinates presented are shown with the percentage of variation explained by them. The points represent 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**)

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

430 **Methylation level in Hsp70**

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

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

458 **Global methylation profiles**

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

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

490 following the exposure to the stressful environment [36]. For instance, a
491 study on three species of coral demonstrated that DNA methylation vari-
492 ation influences their tolerance to thermal stress and ocean acidification
493 [13].

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

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

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

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

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

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534 **Disclosure statement**

535 The authors declare that they have no competing interests.

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686 **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

687

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

688

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 forward B (FB) with the primer reverse B (RB), and the primer forward 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