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1 ***Dnmt1* has an essential function despite the absence of CpG DNA methylation in the red flour beetle**
2 ***Tribolium castaneum***

3

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24 Abstract

25 Epigenetic mechanisms, such as CpG DNA methylation enable phenotypic plasticity and rapid
26 adaptation to changing environments. CpG DNA methylation is established by DNA methyltransferases
27 (DNMTs), which are well conserved across vertebrates and invertebrates. There are insects with
28 functional DNA methylation despite lacking a complete set of *Dnmts*. But at least one of the enzymes,
29 DNMT1, appears to be required to maintain an active DNA methylation system. The red flour beetle,
30 *Tribolium castaneum*, lacks *Dnmt3* but possesses *Dnmt1* and it has been controversial whether it has
31 a functional DNA methylation system.

32 Using whole genome bisulfite sequencing, we did not find any defined patterns of CpG DNA
33 methylation in embryos. Nevertheless, we found *Dnmt1* expressed throughout the entire life cycle of
34 the beetle, with mRNA transcripts significantly more abundant in eggs and ovaries. A maternal
35 knockdown of *Dnmt1* caused a developmental arrest in offspring embryos.

36 We show that *Dnmt1* plays an essential role in *T. castaneum* embryos and that its downregulation
37 leads to an early developmental arrest. This function appears to be unrelated to DNA methylation,
38 since we did not find any evidence for this modification. This strongly suggests an alternative role of
39 this protein.

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41 1 Introduction

42 Phenotypic plasticity is enabled by epigenetic mechanisms, which help the organism to adapt rapidly
43 to changing environmental conditions without affecting the underlying genotype [1]. One of these
44 mechanisms is DNA methylation, which in insects is associated with a wide variety of phenotypically
45 plastic traits [2,3]. In eusocial insects, caste formation can correlate with differential DNA methylation
46 patterns [4–6]. One of the first examples for functional DNA methylation in insects was the discovery that
47 the downregulation of the methylation machinery in honey bee larvae caused them to develop into a
48 queen-like phenotype, which is naturally established by the feeding on royal jelly [7]. Furthermore, DNA
49 methylation patterns do not only differ between the brains of queens and workers [5], but also within
50 the worker caste, where they have been associated with different tasks performed by the worker bee
51 [8]. Additionally, these epigenetic marks appear to be reversible if the individual has to switch its task [9].
52 Furthermore, in two ant species the same set of genes was differentially methylated according to
53 developmental stage and caste [6]. DNA methylation can also be observed in non-social insects and may
54 contribute to the striking phase polyphenism between solitary and gregariously living migratory locusts
55 [10,11]. On the other hand, it also has been suggested that DNA methylation can have a more general,
56 basic role in insects and it might function in more ubiquitously essential processes, e.g. embryonic
57 development, genomic imprinting and alternative splicing [6,12–15].

58 Insect methylation patterns can be highly diverse [15,16]. In cockroaches and some lepidopterans, DNA
59 methylation can reach levels similar to those observed in vertebrates and plants [15,17]. This is also true
60 for some regions of the genome of the stick insect, but here in addition to gene bodies also repetitive
61 elements are strongly methylated [18]. In contrast, holometabolous insects possess sparser DNA
62 methylation than vertebrates [15,16,19]. Most of these insects also differ from mammals in the location of
63 their methylation marks, as the majority of CpG methylation occurs within gene bodies [6,20,21]. The
64 placement of methyl groups at these mostly intragenic loci could hint at its possible role in alternative
65 splicing [22].

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66 In invertebrates and vertebrates alike, the same conserved set of enzymes adds methyl groups to
67 cytosines followed by a guanine (CpG) [23]. The resulting CpG DNA methylation patterns are therefore
68 symmetrical on both strands of the DNA. In most cases, two DNA methyltransferases (DNMTs) are
69 needed to sustain functional CpG DNA methylation. DNA methyltransferase 1 (DNMT1) is responsible
70 for maintaining pre-existing methylation patterns by methylating hemimethylated DNA after
71 replication, while DNA methyltransferase 3 (DNMT3) establishes new methylation patterns on
72 previously unmethylated cytosines [23]. Previously, it was thought that a third enzyme, DNMT2, was also
73 involved in DNA methylation because of sequence conservation and the fact that it also contains a
74 cytosine methylase domain [24]. However, several studies have shown that this enzyme methylates small
75 RNAs, especially tRNA [24,25]. Furthermore, organisms that lack *Dnmt1* and *3,* but contain *Dnmt2* do not
76 possess a functional DNA methylation system [24,26,27].

77 The distribution of *Dnmt*s in insects is quite diverse. While many insects, especially eusocial species,
78 have a complete set of *Dnmts*, some with an expansion in copies for *Dnmt1*, other insects completely
79 or partially lack *Dnmts* and some also fail to produce functional levels of DNA methylation [15,19,28].
80 Diptera appear to have lost both *Dnmt1* and *Dnmt3* and with it any relevant CpG DNA methylation [15,16].
81 Some insect species including examples from Coleoptera, Hemiptera, Lepidoptera and even social
82 Hymenoptera are lacking a gene encoding for DNMT3 [15,29–31]. Nevertheless, several of them may show
83 levels of DNA methylation, which presented either directly in sequencing studies or were deduced
84 from the CpG depletion in their genomes [15,16,30]. Thus, the question arises, how a functional DNA
85 methylation system can be sustained in the absence of an enzyme able of *de novo* methylation. It has
86 been suggested that DNMT1 could take over this function, but to the best of our knowledge there are
87 no studies demonstrating this.

88 The status of a functional DNA methylation system in the red flour beetle *Tribolium castaneum* has
89 been controversially discussed over the past years. Over evolutionary time, the mutagenic effect of a
90 methyl group attached to a cytosine leads to a depletion of CpGs from the genome [32]. However, the
91 beetles' genes do not exhibit the bimodal distribution of normalized CpG content associated with the

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92 presence of DNA methylation in other insects [4,15]. Additionally, in adult beetles, low-coverage whole-
93 genome bisulfite sequencing (WGBS), which reveals methylation at single-base resolution, could not
94 detect any defined patterns of CpG DNA methylation [19]. On the other hand, using an approach based
95 on methylation sensitive restriction endonucleases, which lacks the sensitivity and specificity of WGBS,
96 Feliciello *et al.* (2013) found evidence for DNA methylation in early embryos, whereas larvae, pupae
97 and adults were found to be mostly devoid of methylation. While this raised the possibility of an active
98 demethylation process in later developmental stages, resembling the epigenetic reprogramming in
99 mammalian embryos [34,35], additional bisulfite sequencing of satellite DNA indicated that these
100 fragments remain heavily methylated throughout the entire lifetime of the individual, even in non-CpG
101 contexts [33]. Furthermore, another study based on bisulfite sequencing also found DNA methylation
102 outside the context of CpG and mostly at CpA [36].

103 The controversial evidence of CpG DNA methylation in *T. castaneum* demands for additional,
104 functional studies in this important insect model organism. In *T. castaneum* we observe phenotypic
105 plasticity in the form of immune priming, *i.e.* a more successful response to a pathogen at a secondary
106 exposure [37]. This immune priming can be maternally and paternally transferred to the offspring [38–40],
107 which makes the involvement of epigenetic modifications in this phenomenon likely. Besides other
108 epigenetic mechanisms, DNA methylation and its facilitating enzymes have been indicated to play a
109 role in the evolution of resistance and the rapid adaption we see during host-parasite co-evolution
110 [41,42]. Therefore, we here investigate the existence of CpG DNA methylation and the role of *Dnmt1* in
111 *T. castaneum* using WGBS and knockdown of *Dnmt1* using maternal RNAi.

112 2 Results

113 2.1 WGBS confirms the absence of CpG methylation in *Tribolium* eggs

114 To detect CpG DNA methylation at single base resolution, we performed WGBS on two replicates of
115 pooled eggs, which gave us a six-fold coverage of the entire genome. The conversion rate of cytosines
116 to uracil by the bisulfite treatment was 99.59 % and 99.58 % for the mapped reads for replicate one
117 and two, respectively. For sites containing at least one unconverted cytosine the mean level of non-

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118 conversion was 0.3 %. Only 1038 partially unconverted CpGs occurred at the same site in both
119 replicates. Overall 0.1 % of the cytosines at these sites were unconverted. Only 84 (8.1 %) of the sites
120 with unconverted cytosines that were observed in both replicates mapped on a chromosome, while
121 the rest was derived from unmapped regions. We also observed a highly significantly negative
122 correlation between the fold coverage and the proportion of unconverted cytosines observed at the
123 same CpG site (rho=-0.925, n=1038, p<0.001, Figure 1). This implies that the cytosines that remain
124 after the bisulfite treatment can be either attributed to incomplete conversion or sequencing errors
125 and therefore no CpG DNA methylation is present in the embryos.

126 ## 2.2 Expression of *Dnmt1*

127 To investigate the expression of *Dnmt1* across the entire life cycle of the beetle we analysed samples
128 of eggs, larvae, pupae and adults via RT qPCR. We used equal numbers of male and female adults and
129 pupae, while sex was not determined for eggs and larvae. All samples examined during this study
130 contained measurable amounts of *Dnmt1* mRNA. The expression of *Dnmt1* was significantly higher in
131 the eggs compared to whole body samples from adult beetles (*Table 1*). In larvae and pupae, we found
132 *Dnmt1* to be expressed at similar levels as in the adult samples (*Table 1*). This suggested that *Dnmt1*
133 might have a function throughout the whole life of the beetle.

134 We dissected virgin beetles of both sexes to investigate the expression of *Dnmt1* in the gonads. Any
135 involvement in transgenerational processes would probably require the expression of the gene in the
136 reproductive tissues so that mRNAs could be passed on to the offspring. In the female samples *Dnmt1*
137 was significantly upregulated in the ovaries, while there was no significant difference in the expression
138 of *Dnmt1* between the testes and whole-body samples in the males (*Table 1*).

139 ## 2.3 Knockdown via maternal RNAi affects development and fecundity

140 To investigate possible functions of *Dnmt1* a knockdown via maternal RNAi was established. Firstly, we
141 confirmed that the injection of *Dnmt1* dsRNA into female pupae led to a significant down-regulation
142 in comparison to RNAi control injections, as measured by RT qPCR in the adult beetles. The treatment

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143 with *Dnmt1* dsRNA significantly reduced the expression of the target gene compared to the RNAi
144 control, while expression did not differ significantly between the untreated and RNAi control (Table *1*).
145 The knockdown was also transferred to the offspring, as the eggs of treated mothers showed
146 significantly reduced amounts of *Dnmt1* dsRNA compared to eggs from the RNAi control (Table *1*).

147 Furthermore, *Dnmt1* knockdown significantly reduced the eclosion rate from pupae to imago (GLMM
148 with binomial data distribution, df=2, X^2 =146.2, p<0.001), suggesting that the protein plays a role in
149 metamorphosis. Following *Dnmt1* knockdown, the eclosion rate three days post injection was 80 %,
150 which was significantly lower than for the RNAi control (90 %) (Tukey's HSD: z=6.93, p<0.001; Figure
151 2a). The naïve control achieved 96 % survival, which is significantly better than the RNAi control
152 (Tukey's HSD: z=-4.74, p<0.001; Figure 2a). This can be explained by the absence of injection wounds
153 in these animals.

154 We determined the fecundity of the treated beetles, by calculating hatching rates for one hundred
155 eggs per treatment and experimental replicate. This showed that the fitness of the females, which had
156 received the *Dnmt1* knockdown treatment was severely affected, as the hatching rate of their offspring
157 was significantly reduced and only 13 % of larvae hatched, compared to 69 % in both controls (GLMM
158 with binomial data distribution, df=2, X^2=274.46, p<0.001; Tukey HSD: *Dnmt1* – RNAi control z=12.7,
159 p<0.001, *Dnmt1* – naïve z=12.8, p<0.001, Figure 2b).

160 To confirm that the observed reduced fecundity was produced by the knockdown of *Dnmt1* and not
161 any potential off-target effect on a different gene, we used a second non-overlapping construct. This
162 construct also caused a significant downregulation in adults (Table *1*) and significantly reduced female
163 fecundity (Kruskal-Wallis df=3, X^2=40.7, p<0.001, Figure 2c) compared to the naïve (Construct a:
164 W=731.5, p<0.001, Construct b: W=675.5, p=0.001) and RNAi control (Construct a: W=553.5, p<0.001,
165 Construct b: W=470, p=0.03). The two controls did not differ significantly from each other (W=301,
166 p=0.44).

167 Additionally, we also investigated the effect of *Dnmt1* expression on male fertility. A knockdown in
168 male pupae significantly reduced *Dnmt1* expression in the adults (Table *1*) but did not reduce their

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169 fertility over the first nine days of mating compared to the RNAi control or naïve group (GLMM with
170 negative binomial data distribution, df=2, X^2=2.3441, p-value=0.31).

171 ## 2.4 *Dnmt1* knockdown disrupts embryonic development

172 We also performed a closer analysis of embryos after maternal knockdown of *Dnmt1*. After twelve
173 days, hardly any larvae had hatched, and we therefore assumed that the eggs had either not been
174 fertilised or that the knockdown was causing an embryonic developmental arrest, which was
175 previously observed in *Nasonia vitripennis* [13]. In order to identify the time point of a potential
176 developmental arrest, we sampled eggs every two hours for the first ten hours after oviposition.
177 According to their developmental status, the eggs were then assigned to one of four categories of a
178 timeline of normal embryonic development. At the two first time points, we did not observe any
179 significant developmental differences between the treatment groups (age 0-2 h: $OLM_{2, 96}$, $\chi 2=0.84$,
180 p=n.s.; age 2-4 h: $OLM_{2, 140}$, $\chi 2=4.65$, p=0.098). But, when the embryos were between four and six
181 hours old, more than 60 % of the eggs of the knockdown treatment fell within the earliest category of
182 development and therefore differ significantly from the two controls (OLM_{107}, $\chi 2=35.71$, $p<0.001$). We
183 observed a similar pattern at the embryonic ages of six to eight hours (OLM_{105}, $\chi 2=55.11$, $p<0.001$) and
184 eight to ten hours (OLM_{144}, $\chi 2=40.76$, $p<0.001$). We therefore conclude that the knockdown of *Dnmt1*
185 causes an early embryonic arrest after first few cleavage cycles (Figure 3).

186 # 3 Discussion

187 Using WGBS, we did not observe any recognizable evidence for CpG DNA methylation in *T. castaneum*
188 embryos. The few candidate methylation marks detected by whole genome sequencing are most likely
189 due to incomplete bisulfite conversion or sequencing errors. Furthermore, there is hardly any overlap
190 in the candidate mCpGs between the two replicates, which leads us to conclude that CpG DNA
191 methylation is absent in two-day old embryos. Our results agree with those of Zemach *et al.*, who were
192 also unable to detect any significant levels of CpG DNA methylation in *T. castaneum* [19]. Yet, two other
193 studies found DNA methylation in all life stages of the beetle. While Song *et al.* (2017) observed a

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194 strong preference for CpA methylation, which because of its asymmetrical nature is very unlikely to be
195 produced by DNMT1, Feliciello *et al.* (2013) found CpG methylation especially on satellite DNA but also
196 spread across the genome of embryos. These differences might be explained by changing methylation
197 patterns during different life stages and possible rapid dynamic changes during embryonic
198 development similar to the epigenetic reprogramming in mammals [34]. Alternatively, they might also
199 result from incomplete bisulfite deamination, which is often linked to the artefactual detection of
200 methylation outside of the canonical CpG context.

201 *T. castaneum*, as several other insect species, lacks *Dnmt3* in its genome and therefore does not
202 possess a complete DNA methylation system [3,29,43]. Nevertheless, some insect species lacking a
203 complete set of *Dnmts*, show detectable DNA methylation including diverse methylation patterns in
204 correlation with environmental changes [15,16,44]. It is still unknown whether *Dnmt1* can take over the
205 function of the missing *Dnmt3* or if other enzymes are responsible for preserving the function.
206 However, the lack of CpG methylation observed in the present study is further supported by the
207 absence of CpG depletion from the beetle's gene set, which is a known evolutionary signal of CpG
208 methylation [32,45]. To ultimately resolve this controversy, further studies are needed to determine
209 whether, where and at which time functional levels of CpG DNA methylation might occur in
210 *T. castaneum*.

211 Our results also show that *Dnmt1* is present and expressed in *T. castaneum* during all life stages, in
212 both sexes and in the reproductive tissues despite the apparent absence of CpG DNA methylation. The
213 expression of *Dnmt1* during the entire life cycle of the beetle hints at a possible function during several
214 life stages. Because ovaries and eggs showed significantly higher relative amounts of *Dnmt1* mRNA,
215 the protein might be especially relevant during reproduction and embryonic development.

216 Both constructs of *Dnmt1* used in our RNAi experiment significantly reduced the levels of *Dnmt1*
217 mRNA. Although the reduction was only to 70% compared to the levels observed in the treatment
218 control, it still produced a strong phenotype in its effect on offspring embryonic development. RNAi
219 treatment in *B. mori* leads to a similar degree of reduction of *Dnmt1* and also decreased hatchability

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220 [44]. A recently described alternatively spliced isoform of *Dnmt1* in *T. castaneum* lacks the C-terminal
221 DNA methylase domain and is thus unable to methylate DNA, but was shown to be predominantly
222 expressed [36]. While the dsRNA constructs used here should cause a knockdown of both isoforms, our
223 gene expression analysis was only able to detect the transcript containing the methylase domain. Thus,
224 the RNAi treatment might affect the predominant shorter isoform to a greater extent.

225 We could show that *Dnmt1* is essential for early embryonic development even in the absence of
226 functional levels of CpG DNA methylation. The knockdown in mothers leads to high lethality in the
227 offspring embryos within the first hours after oviposition. Similarly, the maternal knockdown of one
228 copy of *Dnmt1* in *N. vitripennis* also lead to a developmental arrest in the embryos but at a later time
229 point [13]. Additionally, fecundity in brown planthoppers was strongly decreased after *Dnmt1*
230 knockdown [46]. DNMT1 appears to also have an essential role in metamorphosis of *T. castaneum* as a
231 reduction during the pupal stage decreased the rate of eclosion. It is therefore reasonable to assume
232 that DNMT1 has additional functions in *T. castaneum* and maybe also in other insects, including those
233 possessing a DNA methylation machinery.

234 Alternative, non-catalytic functions of *Dnmt1* have been suggested [47]. The DNA-binding function could
235 be intact in the absence of methylase domain, still enabling the enzyme to fulfil other tasks [36]. This is
236 supported by the presence of a large N-terminal domain without methylase function but with many
237 molecular binding patterns [47]. For example, specific motifs of the N-terminal domain have been shown
238 to regulate the transcription of E-cadherin in humans without the association with any changes in
239 methylation patterns [48]. Further studies in vertebrates indicate that DNMT1 binding to histone
240 modification enzymes is involved in modulation of gene expression [49,50]. Additional support comes
241 from the clawed frog *Xenopus laevis*, for which *Dnmt1* expression is also essential during embryonic
242 development [51]. Here, the protein silences genes by directly repressing transcription independent of
243 its catalytic function [52]. The downregulation of *Dnmt1* leads to the premature activation of gene
244 expression before the mid-blastula transition without any larger changes in methylation patterns [52].
245 This may indicate that also in insects including *T. castaneum* the sufficient provision of maternal *Dnmt1*

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246 transcripts ensures the correct timing of the maternal to zygotic transition of mRNA transcription and
247 thereby prevents embryonic developmental arrest and death. The results of this study emphasise a
248 presumably non-catalytic but essential role of *Dnmt1*, which needs to be investigated in more detail.

249 # 4 Methods

250 ## 4.1 Model organism

251 We used beetles from the Georgia 2 (GA-2) line for WGBS, since the genome sequence of *T. castaneum*
252 was produced from that line [29]. Expression analysis and knockdown experiments were carried out with
253 individuals from the San Bernadino (SB) line originating in the Sokoloff laboratory (Tribolium Stock
254 Center, California State University, San Bernardino, USA). Beetles were kept on organic wheat flour
255 (type 550) with 5 % brewer's yeast at a population size of more than 200 individuals. They were
256 maintained at 30 °C and 70 % humidity at a 12h-light/12h-dark cycle.

257 ## 4.2 Whole genome bisulfite sequencing

258 WGBS was performed on two replicates each from 300-400 µl of eggs. We collected eggs after a 24
259 hours oviposition period from a population of about 1000 individuals of the GA-2 strain. Samples were
260 incubated at 30 °C with 70 % humidity for an additional 24 h, then frozen in liquid nitrogen, and stored
261 at -80 °C.

262 Per sample 5 µg of high molecular weight DNA were used for fragmentation using the Covaris S2 AFA
263 System in a total volume of 100 µl. The fragmentation (parameters: duty cycle: 10 %; intensity: 5;
264 cycles/burst: 200; cycles: 3) was run for a total fragmentation time of 180 s. Fragmentation was
265 confirmed with a 2100 Bioanalyzer (Agilent Technologies) using a DNA1000 chip. The fragmented DNAs
266 were concentrated to a final volume of 75 µl using a DNA Speed Vac. End repair of fragmented DNA
267 was carried out in a total volume of 100 µl using the Paired End DNA Sample Prep Kit (Illumina) as
268 recommended by the manufacturer. For the ligation of the adaptors, the Illumina Early Access
269 Methylation Adaptor Oligo Kit and the Paired End DNA Sample Prep Kit (Illumina) were used, as

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270 recommended by the manufacturer. For the size selection of the adaptor-ligated fragments, we used
271 the E-Gel Electrophoresis System (Invitrogen) and a Size Select 2 % precast agarose gel (Invitrogen).
272 Each fragmented DNA was loaded on two lanes of the E-gel. Electrophoresis was carried out using the
273 "Size Select" program for 16 min. According to the standard loaded (50 bp DNA Ladder, Invitrogen),
274 240 bp fragments were extracted from the gel, pooled, and directly transferred to bisulfite treatment
275 without further purification. For the bisulfite treatment we used the EZ-DNA Methylation Kit (Zymo)
276 as recommended by the manufacturer with the exception of a modified thermal profile for the bisulfite
277 conversion reaction. The conversion was carried out in a thermal cycler using the following thermal
278 profile: 95 °C for 15 s, 50 °C for 1 h, both steps repeated 15 times, 4 °C for at least 10 min. The libraries
279 were subsequently amplified, using the Fast Start High Fidelity PCR System (Roche) with buffer 2, and
280 PE1.1 and PE2.1 amplification primers (Illumina). PCR thermal profile: 95 °C for 2 min, 95 °C for 30 s,
281 65 °C for 20 s, 72 °C for 30 s, then repeat from step 2, 11×, 72 °C for 7 min, hold at 4 °C. PCR reactions
282 were purified on PCR purification columns (MinElute, Qiagen) and eluted in 20 µl elution buffer
283 (Qiagen).

284 Paired-end sequencing was performed on an Illumina HiSeq 2000 system with read lengths of 105 bp
285 and an average insert size of 240 bp. Reads were trimmed and mapped with BSMAP 2.5 [53] using the
286 assembly version 3.0 of the *T. castaneum* genome (https://www.hgsc.bcm.edu) as a reference
287 sequence. Duplicates were removed using the Picard tool (http://broadinstitute.github.io/picard).
288 Methylation ratios were determined using a Python script (methratio.py) distributed together with the
289 BSMAP package.

290 For every site containing a CpG, we calculated the fold coverage and the proportion of cytosines at
291 each site that had not been converted by the bisulfite treatment. We also computed which cytosines
292 that were partially unconverted occurred in both replicates and combined this data. Finally, we
293 checked whether there is a correlation between proportion of unconverted cytosines and fold
294 coverage.

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295 ### 4.3 Gene Expression Analysis

296 We measured the expression of *Dnmt1* in different life history stages (eggs, larvae, pupae, adults) as
297 well as in the reproductive organs of the beetle using quantitative real-time PCR (RT qPCR). At the time
298 point of collection, around 200 µl of eggs were used per sample, while for all other samples material
299 from five individuals was pooled, immediately shock frozen in liquid nitrogen and stored at -80 °C. We
300 analysed four replicates for each life history stage as well as for the gonad and whole-body samples.

301 The applied RNA extraction protocol combines phenol/chloroform lysis and extraction with the
302 purification via spin columns from the SV Total RNA Isolation System (Promega) and was performed as
303 described in Eggert et al. (2014). We performed reverse transcription into complementary DNA (cDNA)
304 with the RNA-dependent DNA polymerase SuperScript III and the use of oligo(dT)$_{18}$ primers
305 (Invitrogen). The reaction started with 100 ng RNA and produced a final volume of 10 µl. The samples
306 were stored at 4 °C for up to one week. For all samples, we measured the expression of *Dnmt1* relative
307 to the expression of two housekeeping genes the *ribosomal protein L13a* (*RpL13a*) and *ribosomal*
308 *protein 49* (*rp49*). All primers were designed to cross exon-intron boundaries and their quantification
309 efficiency (E) was calculated using a serial dilution curve (Supplementary Table S1).

310 We applied qPCR using the LightCycler® 480 Real-Time PCR System (Roche) and KAPA SYBR® FAST qPCR
311 Light Cycler 480 reaction mix (PEQLAB). Prior to the run of the assay, we diluted the cDNA 1:10. All
312 experiments were carried out with two technical replicates per sample. Crossing points (Cp) for each
313 technical replicate were calculated and used in further analysis if the standard deviation (STD) between
314 the two technical replicates was below 0.5.

315 ### 4.4 Knockdown via parental RNAi

316 To accomplish a knockdown of *Dnmt1* via parental RNAi, we designed a construct for double stranded
317 RNA (dsRNA). DsRNA complimentary to the *Asparagin synthetase A* gene (*AsnA*) from *Escherichia*
318 *coli* served as a treatment control [55]. We produced both dsRNA constructs using EcoRV digested
319 pZErO™-2 vector (Invitrogen) and *in vitro* transcribed into dsRNA with the T7 RNA polymerase

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320 based T7 MEGAscript Kit (Ambion). iBeetle-Base provided us with the sequence for the second non-
321 overlapping construct, which we used in order to be able to exclude possible off-target effects
322 (Schmitt-Engel et al. 2015, http://ibeetle-base.uni-goettingen.de/details/iB_08496). For this we
323 obtained the dsRNA from EupheriaBiotech.

324 For the RNAi procedure, we treated female and male pupae with either *Dnmt1* dsRNA, *AsnA* dsRNA or
325 left them naïve, *i.e.* untreated. For the injections, we adjusted the concentration of dsRNA to
326 1,800 ng/µl. Prior to the injections, three-day old pupae were collected and glued with the end of their
327 abdomen to microscopic slides with Fixogum (Marabu). The injections were carried out with a nano
328 injector (FemtoJet, Eppendorf) using borosilicate glass capillaries (100 mm length, 1.0 mm outside
329 diameter, 0.021 mm wall thickness; Hilgenberg). Pupae were injected with the dsRNA solution laterally
330 between the second and third segment of the abdomen. We stopped the injections when the pupae
331 stretched, due to the increasing turgor.

332 The pupae were left on the glass slides in Petri dishes until they eclosed one to five days later. After
333 eclosion individual survival was recorded and the surviving beetles could mature for four days. At this
334 point, we randomly sampled individuals for either expression analysis or mating and egg production.
335 Due to large sample sizes required in the experiment involving embryo staining, we conducted these
336 injections in three consecutive experimental blocks. We confirmed the success of the knockdown by
337 performing expression analysis on four pools of three to five individuals per treatment and block.
338 Additionally, we measured the *Dnmt1* mRNA amount present in the eggs, by performing RT qPCR on
339 one pool of 150-200 µl eggs per each of the three experimental blocks and treatment groups. At the
340 time point of RNA extraction eggs of each sample were between 45 minutes and 16 hours old.

341 ## 4.5 Fitness costs of the knockdown

342 To estimate potential fitness costs of the *Dnmt1* knockdown treatment we collected one hundred eggs
343 from each of the treatment groups and controls, individualised them and counted live larvae twelve
344 days later. For the second non-overlapping dsRNA construct (construct b) fitness costs were examined

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345 in a different way. The construct from the previous experiment (construct a) was also used here. We
346 set up single mating pairs (n=23-30) and let them mate for 24 hours. Then live larvae were counted
347 twelve days later for each pair. Besides the two dsRNA constructs, a RNAi control and a naïve group
348 were included.

349 To measure the fertility of the males after RNAi treatment, individual mating pairs with untreated,
350 virgin females of the same age were formed four days post eclosion. The pairs were put on to new
351 flour after 24 hours and then again, every three days for the next nine days. Two weeks after the
352 oviposition living larvae were counted for each pair (n=20-26).

353 4.6 Embryonic development after maternal knockdown

354 To determine a possible effect of the maternal knockdown on their offspring, we observed the
355 embryonic development via DNA staining. For this we collected eggs every two hours over a period of
356 ten hours, which enabled us to look at five individual time points after oviposition. For the fixation, we
357 washed the eggs with water and treated them with 25 % DanKlorix (Colgate –Palmolive), which
358 contains 5% sodium hypochlorite to remove the chorion. The eggs were submerged in heptane and
359 fixed with 4 % formaldehyde in phosphate buffered saline (PBS). Vigorous shaking in methanol led to
360 the removal of the vitelline membrane. Finally, the methanol was replaced with 95% ethanol and the
361 fixed embryos were stored at -20 °C.

362 We determined the developmental stage of the previously fixed embryos through staining with 4',6-
363 diamidino-2-phenylindole (DAPI) (Carl Roth). After incubating the embryos in PBS with 0.5 µg/ml DAPI
364 for five minutes, they were washed with PBS twice and mounted to a microscopy glass slide using
365 Fluoroshield histology mounting medium (Sigma-Aldrich). A fluorescence inversion microscope
366 (Observer Z1, Zeiss) with an attached fluorescence camera (AxioCamMR3, Zeiss) was used for taking
367 pictures at 200x magnification. We ascertained embryonic development by assigning each picture to
368 one of four categories. These categories were formed from a time line of naïve development over the

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369 first ten hours after oviposition (cat.I=ca. 1h, cat.II=ca. 3h, cat.III=ca. 5h, cat.IV=ca. 8h), which was
370 established prior to this experiment.

371 4.7 Statistical analysis

372 If not stated otherwise, all statistical analyses were performed in R (R Development Core Team 2008,
373 version 3.4.0) under RStudio version 0.99.467 [58] using packages lme4 [59] and MASS [60] when performing
374 generalised linear mixed effects models (GLMMs). The correlation between fold coverage and
375 proportion of unconverted cytosines was tested using a Spearman rank test.

376 All expression data of *Dnmt1* were analysed and tested for significant differences using the relative
377 expression software tool REST2009 (Qiagen ; Pfaffl, Horgan, & Dempfle, 2002) as previously described
378 [54]. Bonferroni correction for multiple testing was applied to the results if the control group was
379 compared to more than one treatment group.

380 For examining the eclosion and therefore survival after injection of the females in the knockdown
381 experiments a GLMM with binomial error distribution was applied to the data. We investigated the
382 effect of the knockdown on maternal fitness by testing hatching rates of larvae for differences in a
383 GLMM with binomial error distribution followed by a Tukey's HSD test. Fecundity of individual pairs
384 after maternal knockdown using both *Dnmt1* RNAi constructs was tested with a Kruskal-Wallis test,
385 then pairwise comparisons were performed with a Wilcoxon test and p values were adjusted according
386 to Benjamini & Hochberg (1995). Male fertility after knockdown was analysed with a generalised linear
387 model (GLM) with negative binomial error distribution.

388 The embryonic development after maternal knockdown was tested with an ordinal logistic model
389 (OLM) and a post-hoc maximum likelihood test to determine, whether the distribution of embryos to
390 the four developmental categories was significantly different between the maternal treatments. This
391 was done for each observed time point separately. Analyses were performed with JMP version 8 (SAS
392 Institute Inc).

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393 5 Data availability.

394 The datasets generated during and/or analysed during the current study are available from the
395 corresponding author on reasonable request.

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531

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534 8 Author contributions

535 Conceived and designed study: NS, IW, MDdB, FL, JK. Carried out lab work: NS, IW, JE. Carried out
536 sequencing: GR. Analysed data: NS, GR. Wrote manuscript: NS with comments from all other authors.

537 9 Competing Interest Statement

538 The authors declare no competing interests.

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539 ## 10 Tables

540 Table 1 Gene expression of *Dnmt1* normalised over the expression of two housekeeping genes.

	relative expression of *Dnmt1*	**95% C. I.**	**n**	**p value**
Life stages (relative to adults)				
eggs	**1.660**	1.027 - 3.144	4	**0.004**
larvae	0.818	0.511 - 1.555	8	0.063
pupae	0.794	0.390 - 1.784	8	0.176
adults	1.000	0.535 - 1.878	8	0.997
Gonads (relative to whole-body samples)				
male	1.382	1.008 - 1.828	4	0.056
female	**1.841**	1.434 - 2.226	4	**0.018**
RNAi (relative to treatment control)				
Females				
Dnmt1 construct a	**0.680**	0.301 - 1.226	11	**0.002**
naive	1.012	0.543 - 1.883	12	0.904
Eggs				
Dnmt1 construct a	**0.442**	0.226 - 0.753	3	**0.025**
naive	1.062	0.458 - 2.062	3	0.869
Females				
Dnmt1 construct a	**0.646**	0.553 - 0.752	4	**0.011**
Dnmt1 construct b	**0.703**	0.536 - 0.906	4	**0.021**
naive	1.007	0.865 - 1.199	4	0.856
Males				
Dnmt1 construct a	**0.686**	0.542 - 0.822	3	**<0.001**
naive	1.062	0.853 - 1.312	3	0.458

541

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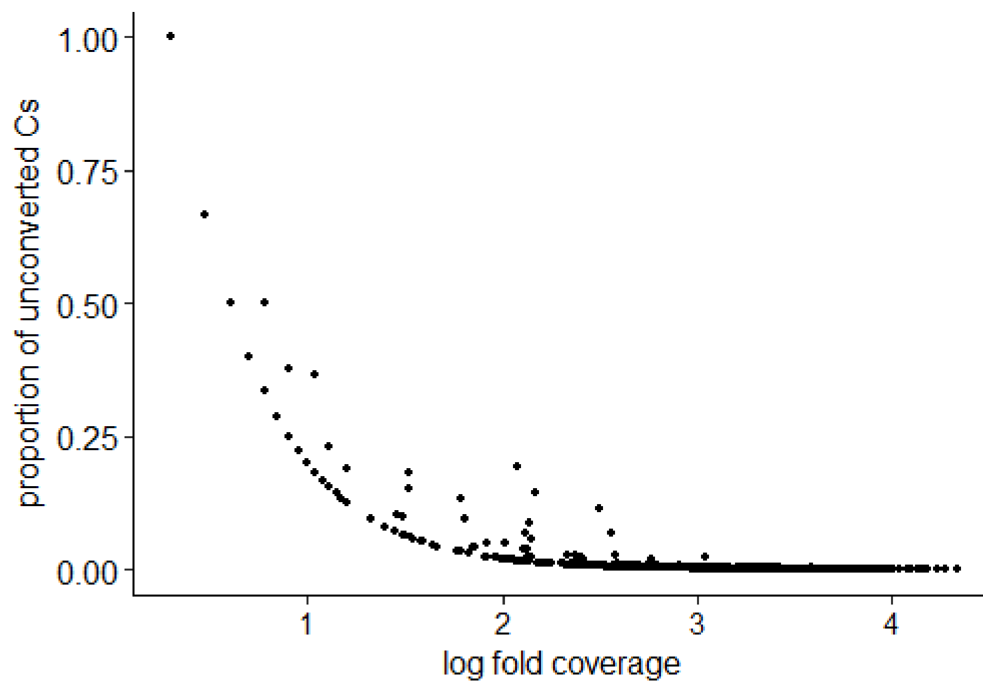
542 11 Figures



543

544 *Figure 1* Correlation between the proportion of unconverted cytosines that were observed in both replicates of the WGBS
545 and the log fold coverage at each site (Spearman rank, rho=-0.925, n=1038, $p<0.001$).

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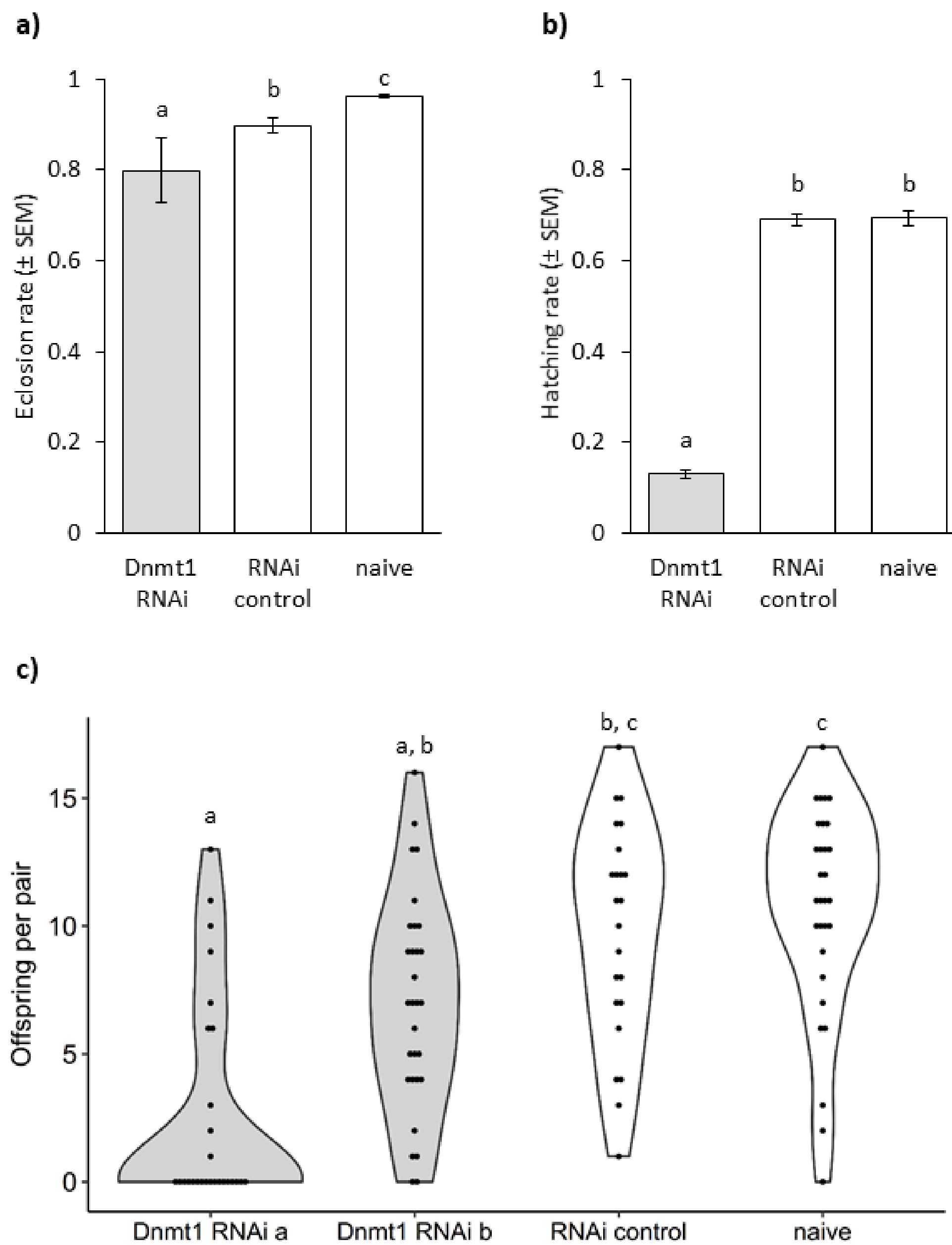

546

547 *Figure 2* ***Phenotypic effects of maternal RNAi*** **a)** Eclosion and survival rate (± SEM) of female pupae after *Dnmt1* RNAi
548 treatment. Three replicates with 240-270 individuals per treatment and block. **b)** Larval hatching rates (±SEM) after mothers
549 received *Dnmt1* RNAi treatment. Three replicates with 100 individualized eggs per treatment and block. **c)** Offspring produced
550 by single mating pairs (n=26-30) after maternal RNAi treatment (*Dnmt1* RNAi construct a or b, RNAi or naïve control). Letters
551 indicate significant differences.

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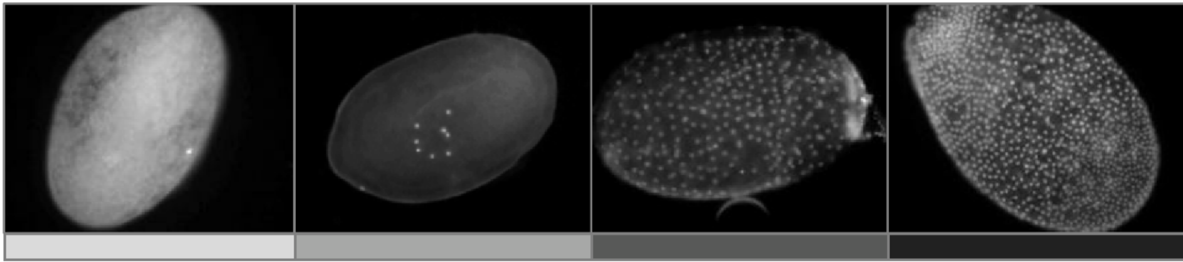
a)



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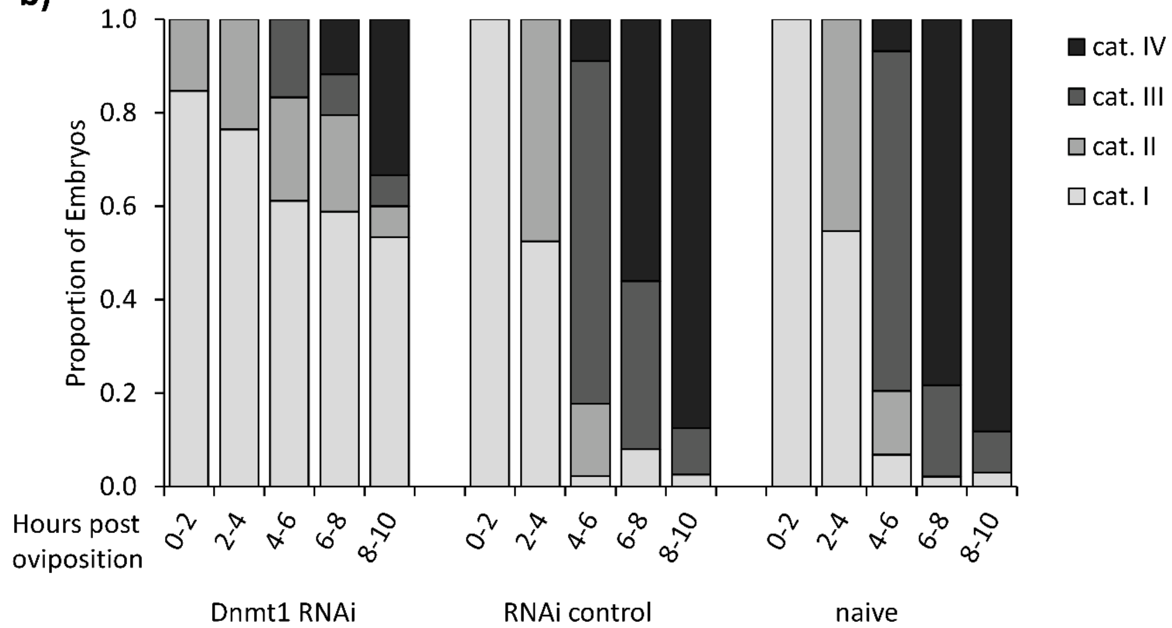

552

553 *Figure 3* ***Dnmt1 knockdown and embryonic development*** **a)** Timeline of naïve embryonic development. Shown is (left to
554 right) a representative example for each of the following age categories: cat. I (ca. 1h), cat. II (ca. 3h), cat. III (ca. 5h), cat. IV
555 (ca. 8h) after oviposition. Eggs were produced by untreated animals. **b)** Phenotypes of embryos after maternal RNAi. Eggs
556 from the treatment and controls (*Dnmt1* RNAi, RNAi control and naïve) and five age groups (hours post oviposition) were
557 sorted according to their developmental status into one of four categories established from the timeline of naïve
558 development.