1 **Title**

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3 Gene-regulatory independent functions for insect DNA methylation

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5 **Short Title**

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7 Functional DNA methylation in an insect

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Abstract

The function of cytosine (DNA) methylation in insects remains unknown. Here we provide evidence for the functional role of the maintenance DNA methyltransferase 1 (*Dnmt1*) in an insect using experimental manipulation. Through RNA interference (RNAi) we successfully post-transcriptionally knocked down *Dnmt1* in ovarian tissue of the hemipteran *Oncopeltus fasciatus* (the large milkweed bug). Individuals depleted for *Dnmt1*, and subsequently DNA methylation, failed to reproduce. Eggs were inviable and declined in number, and nuclei structure of follicular epithelium was aberrant. Depletion of DNA methylation did not result in changes in gene or transposable element expression revealing an important function of DNA methylation seemingly not contingent on gene expression. Our work provides direct experimental evidence for a functional role of *Dnmt1* and DNA methylation independent of gene expression in insects.

Introduction

DNA methylation in insects has been hypothesized to play numerous functional roles including polyphenism, diapause, longevity, and social behavior and caste differentiation [1–21]. However, these hypotheses are based primarily on correlational studies; for example, there is evidence that the dependence of social behavior and caste differentiation on DNA methylation is not absolute [11–13]. Comparative epigenomic studies have provided valuable insights into patterns of DNA methylation across most insects, but have offered limited insights into its functional significance.

In animals DNA methylation typically occurs at CG sites, which is established by DNA methyltransferase 3 (*Dnmt3*), and subsequently maintained by DNA methyltransferase 1 (*Dnmt1*). Many insects possess *Dnmt1* and *Dnmt3;* however, there is variation in presence-absence and copy number of these DNA methyltransferases, and levels and patterns of DNA methylation [12, 13]. In many insects DNA methylation is localized to moderately and constitutively expressed genes that are highly conserved between species. This has led some to the hypothesis that DNA methylation functions in transcriptional regulation [6, 15, 22, 23]. However, functional tests of DNA methyltransferases in insects are limited to the post-transcriptional knock down of *Dnmt3* in *Apis mellifera*, which is associated with modest reductions in DNA methylation, differential gene expression, and alternative splicing [24]. Given the variation in mechanisms that establish and maintain DNA methylation and limited genetic and mutant studies, the function of insect DNA methyltransferases, and DNA methylation itself, is obscure.

Here we show that DNA methylation is maintained by *Dnmt1*, is essential and is not associated with transcription in the hemipteran *Oncopeltus fasciatus* (the large milkweed bug) [25, 26]. Post-transcriptional knockdown of *Dnmt1* led to reduced egg viability, fecundity, and aberrant follicular epithelium, and thus failure to produce a successive generation. Despite finding levels of methylated CG (mCG) within coding regions reduced by 83.55%, we found no evidence for DNA methylation directly affecting transcription. Our results suggest *Dnmt1* plays an important role in reproduction in *O. fasciatus* that is mediated by a gene-regulatory independent function of DNA methylation. *Oncopeltus fasciatus* represents a fruitful model species for functional

68 studies of DNA methylation, and continuation of studies in this system will unravel the
69 insect epigenome and its functional consequences.
70
71 **Results**
72
73 ***Dnmt1* is necessary for reproduction.** To assess the function of *Dnmt1*, double-stranded
74 RNA (dsRNA) targeting *Dnmt*1 (ds-*dnmt1*) (S1 Fig, and S1 and S2 Tables) was injected
75 between the abdominal sternites of virgin *O. fasciatus* females. Maternal dsRNA
76 injection has been used in *O. fasciatus* to post-transcriptionally knockdown expression in
77 embryos developing from eggs laid by injected females [27] and is known to reduce
78 expression of embryonically expressed genes. A control injection of double-stranded *Red*
79 (ds-*red*) or buffer was used to confirm that there was no technical affect associated with
80 RNAi treatment. The effects of RNAi treatment on gene expression was assessed in gut,
81 head, thorax and ovary tissues 10 days after mating to untreated *O. fasciatus* males. The
82 post-transcriptional knockdown reduced *dnmt1* mRNA expression in all examined tissues
83 (Fig 1A, and S2 Fig and S2 Table). Moreover, there was little variation in expression
84 between biological replicates in ds-*dnmt1*-injected and control individuals (Fig 1A).
85 Taken together, treatment with ds-*dnmt1* specifically and reliably knocked down *Dnmt1*
86 transcripts.
87
88 **Fig. 1. *Dnmt1* is required for reproduction in *O. fasciatus*.** (A) Assessment of RNAi
89 treatment targeting *Dnmt1* using qRT-PCR demonstrates successful reduction in
90 transcription in ovaries compared to control. Dots indicate mean expression level, and

error bars indicate standard error of the mean. (B) Parental RNAi injection with ds-*dnmt1* significantly affected the development of eggs laid by injected females compared to eggs laid by control females. Dots indicate mean expression level, and error bars indicate standard error of the mean. (C) Whole ovaries from females removed 12-14 days post-injection (i and ii). In control females (i), mature oocytes can be seen collecting in the lateral oviduct (Od). In ds-*dnmt1* females (ii), no mature oocytes are apparent, but the oviduct has filled with a yolk-like substance. Scale bar equals 1 mm in (i) and (ii). High magnification of the follicular epithelium surrounding a maturing oocyte (iii and iv). Nuclei are stained with Hoechst 33258 and artificially colored turquoise. Nuclei from control follicular epithelium (iii) are round and regular in shape. Nuclei from ds-*dnmt1* females (iv) are highly irregular in shape.

Post-transcriptional knockdown of *Dnmt1* affects egg development and viability. In the early stages following injection, females injected with ds-*dnmt1* did not lay fewer eggs (F = 2.91, df = 1; p = 1.1e-01) (S3 Fig). Although eggs laid by females injected with ds-*dnmt1* within the first 8 days post-injection looked typical, they were significantly less likely to develop than the eggs laid by control females (χ^2 = 8.470; df = 1; p = 4.0e-03). Furthermore, a mean of 93% of the eggs laid by control females initiated development whereas only a mean of 4% of the eggs laid by ds-*dnmt1* females initiated development (Fig 1B). Although eggs laid by the control females that initiated development were viable and hatched, the few eggs laid by ds-*dnmt1*-injected females that initiated development were not viable and failed to hatch.

Post-transcriptional knockdown of *Dnmt1* affects egg production following the first 10-day post-injection. By 10 to 12 days, females injected with ds-*dnmt1* have mainly stopped laying eggs. While ovaries dissected from control females at this stage have intact eggs in the oviduct, the oviducts of ds-*dnmt1*-injected females are either empty or filled with a mass of what appears to be yolk (Fig 1C), indicating a fault in production of a functional chorion. Analysis of ovarian cell structure indicates that knockdown of *Dnmt1* transcripts affects the cell structure of the follicular epithelium, which is responsible for production of the chorion and vitelline envelope. Nuclei of the follicular epithelium in *Dnmt1* post-transcriptional knock downed females are aberrant and fewer in number than that of the control females (Fig 1C). Therefore, both through disruption of embryonic development from eggs produced within a week of injection and cessation of egg production, reproduction is compromised following knockdown of *Dnmt1* transcripts, preventing a successive generation.

Post-transcriptional knock down of *Dnmt1* successfully and severely reduces mCG in ovaries. The successful knockdown of *Dnmt1* transcripts in gut, head, thorax and ovaries prompted the evaluation of the consequences on DNA methylation using whole genome bisulfite sequencing (WGBS). We used a low coverage sequencing approach, which is sufficient for the detection of changes in bulk levels of DNA methylation [28]. Although *Dnmt1* mRNA expression was reduced in all tissues, DNA methylation was only reduced in ovary tissue (Fig 2A–C and S2 Fig). The reduction specifically in ovaries is likely due to the higher rate of cell division in comparison to cells in other tissues

surveyed, which would facilitate the passive of loss of DNA methylation in the absence of *Dnmt1*.

Fig. 2. *Dnmt1* is required for mCG in *O. fasciatus*. (A) Level and genomic location of mCG between hemi– and holometabolous insects, and *D. pulex*, and RNAi treatment targeting *Dnmt1* in *O. fasciatus*. (B) Levels of mCG across gene bodies and one kilobase pairs (1kb) flanking sequence of hemi– and holometabolous insects, and *D. pulex*. Also shown are the levels of mCG across gene bodies for *O. fasciatus* ds-*dnmt1*. (C) Density plot representation of mCG for RNAi treated *O. fasciatus* and species investigated in this study.

High coverage single-base resolution DNA methylomes from ds-*dnmt1* and control ovaries were generated to understand the impact of the loss of *Dnmt1*. Greater than 75% reductions of mCG were observed across the genome, consistent with the decrease observed in the low coverage experiments (S2 Fig). The presence of symmetrical mCG – DNA methylation occurring on both DNA strands at a CpG site – is indicative of the presence of a functional *Dnmt1*. In control individuals methylation at CpGs is highly symmetrical and highly methylated. However, even though knockdown of *Dnmt1* significantly reduces methylation in ovaries, a minority of CpG sites remain symmetrically methylated (Fig 2C). This suggests that there are some cells within ovaries that have wild-type methylomes. The lack of a complete loss of mCG is expected due to the injection of fully developed individuals and hence the presence of fully methylated genomes that existed prior to the injection of dsRNA.

158 **Comparative epigenomic analysis of the *O. fasciatus* methylome with other insects.**
159 The *O. fasciatus* methylome revealed much higher levels of mCG throughout the
160 genome, similar to other hemimetabolous insects, when compared to holometabolous
161 insects (Fig 2A and B). DNA methylation of gene bodies is found in *O. fasciatus* similar
162 to other insects that possess DNA methylation, however, the pattern of mCG within
163 exonic regions of hemi– and holometabolous insects is distinct (Fig 2B). Higher levels of
164 mCG are towards the 5' end compared to the 3' end of coding regions in holometabolous
165 insects. This distribution resembles the most recent common ancestor of all insects
166 (crustaceans) represented by *Daphnia pulex*. Hemimetabolous insects, which include *O.*
167 *fasciatus,* have a more uniform distribution of mCG across coding regions, and higher
168 levels towards the 3' end of coding regions. Previous studies have demonstrated that CG-
169 methylated genes are often conserved within insects. We identified 39.31% (N = 7,561)
170 CG-methylated genes in *O. fasciatus* and 85.99% of these are CG-methylated in at least
171 one other insect species investigated (S3 Table). Therefore, even though the pattern of
172 DNA methylation within gene bodies of hemimetabolous insects is distinct compared to
173 holometabolous insects, the targeting of specific genes is conserved. Thus, the reductions
174 of mCG across gene bodies in ds-*dnmt1* individuals provides an apportunity to study its
175 potential function.
176
177 **Decreased levels of mCG are not associated with transcriptome-wide changes in**
178 **gene or transposable element expression in ovaries.** We performed RNA-seq analysis
179 from ovary and other tissues in ds-*dnmt1* and control individuals to better understand the
180 relationship between gene expression and DNA methylation. No relationship between

discrete mCG levels and gene expression was observed and this was consistent in other insects that we investigated (Fig 3A, and S4 Fig and S4–7 Tables). Furthermore, no correlation between continuous mCG levels and gene expression was observed (Fig 3B, and S4 Fig and S4–7 Tables). Interestingly, genome-wide relationships between DNA methylation and gene expression were not impacted by the knockdown of *Dnmt1* transcripts (Fig 3B).

Fig. 3. Loss of mCG in *O. fasciatus* ovaries has a limited effect on transcription. (A) Gene expression level for deciles of increasing mCG (1–10) and unmethylated genes (UM). Error bars represent 95% confidence interval of the mean. (B) Regression of gene expression against a continuous measure of mCG with > 0 FPKM for the same set of genes that are CG-methylated in *O. fasciatus* control, but unmethylated in *ds-dnmt1*. Raw *p* values are provided for each regression, and significance or non-significance (NS) is indicated in brackets following Bonferroni correction. (C) Combinational overlap of genes that are differential CG-methylated and expressed, and similarly CG-methylated and expressed between *O. fasciatus ds-dnmt1* and control. Gene groups: Differentially Methylated Gene (DMG), Differentially Expressed Gene (DEG), Similarly Methylated Gene (SMG)/UnMethylated Genes (UMG), and non-Differentially Expressed Gene (non-DEG). (D) A heatmap showing gene expression changes for genes that are differentially CG-methylated between *O. fasciatus* ds-*dnmt1* and control. Expression was standardized by the highest value per gene per biological replicate to produce a Relative Fragments Per Kilobase of transcript per Million (RFPKM) value. RFPKM were clustered using a hierarchical clustering method.

A maximum of 264 differentially expressed genes (DEG) were observed between *O. fasciatus* ds-*dnmt1* and control ovaries (S5 Table). *Dnmt1* was down-regulated in all ds-*dnmt1* samples and no detection of the *de novo* DNA methyltransferase *Dnmt3* was observed. Additionally, no DEGs are observed between *O. fasciatus* ds-*dnmt1* and control for gut, head and thorax (S8 Table). Of genes with differences in DNA methylation between ds-*dnmt1* and control ovaries (N = 6,590), the majority (N = 6,484; 98.39%) had no changes to gene expression (Fig 3C, and S4 and S5 Tables). Furthermore, mCG was always reduced in ds-*dnmt1* compared to control ovaries in the 6,484 gene set. Despite genes being unmethylated (UM) in ds-*dnmt1* ovaries, changes to gene expression occurred in both directions for the 106 differentially DNA methylated and expressed genes (Fig 3D). Even for genes that by definition are unmethylated (N = 5,982) or similarly CG-methylated (N = 21) in ds-*dnmt1* and control, a similar proportion (N = 6,003; 97.53%) was observed to have no difference in gene expression. The lack of an association between DNA methylation and gene expression is further supported when applying more stringent thresholds to the definition of CG-methylated and unmethylated genes (S5 Fig). Our observations support a trivial role of DNA methylation in gene expression if any at all.

The function of DNA methylation in *O. fasciatus* might lie within genome defense – the transcriptional regulation of repetitive DNA and transposons – as its genome is composed of a nontrivial amount of repetive DNA and transposons (6.21%) (S9 Table). However, as in genes, reduction of mCG in ds-*dnmt1* compared to control ovaries was found across the bodies of the TEs, and were not associated with a genome-

wide increase in expression (Fig 4A–C, and S6 Fig and S10 Table). This further supports a trivial role of DNA methylation in transcriptional regulation of loci.

Fig. 4. Large-scale reactivation of TEs did not follow severe reductions of mCG in *O. fasciatus* ovaries. (A) CG methylation levels for the top ten most abundant TEs of ≥ 500 bp in the *O. fasciatus* genome. The dashed lines correspond to the intergenic mCG level of *O. fasciatus* ds-*dnmt1* and control. (B) Levels of mCG across the bodies and 1kb flanking sequence of TEs for a single representative of DNA transposons (Chapaev), LTR retrotransposons (Gypsy), and non-LTR retrotransposons (Jockey). (C) Expression quantified as RPKM for the top ten most abundant TEs of ≥ 500 bp. The subset presents the RPKM distribution when all TEs are considered within ds-*dnmt1* and control ovaries.

Discussion

DNA methylation has been implicated to play numerous roles in insects [1–21], however, its exact function remains uncertain. In this study we observed that post-transcriptional knockdown of *Dnmt1* negatively impacts reproduction through a decrease in egg development and egg production in *O. fasciatus*.

Post-transcriptional knockdown of *Dnmt1* transcripts impacted the maternal somatic gonad and egg maturation. The follicular epithelial cells undergo multiple rounds of mitosis during oogenesis [29]. Oocytes that were in the early stages of maturation would be the most effected, as their follicular epithelium would passively lose DNA methylation with each successive round of mitotis. Loss was not compensated by the *de*

249 *novo* DNA methyltransferase *Dnmt3* as expression was not observed in ovaries. The loss
250 of function of follicular epithelial cells, which includes production of the chorion and
251 vitelline membrane, could explain the loss of oocyte integrity.

252 The knockdown of *Dnmt1* transcripts in *O. fasciatus* ovaries subsequently did not
253 lead to transcriptome-wide changes in gene expression. No correlation between mCG and
254 gene expression in *O. fasciatus* ovaries and other insects was observed (Fig 3 and S4 Fig)
255 [6, 13]. Hence, the importance of mCG in *O. fasciatus* might lie within the orchestration
256 of genomic structure during cell replication, gametogenesis, or cell types not examined in
257 our study.

258 DNA methylation might be required for proper mitosis, and the segration of sister
259 chromatids into their respective daughter cells, as there does appear to be aberrant nuclei
260 structure in ds-*dnmt1* compared to control ovarian cells (Fig. 1C). This phenotype could
261 be the result of epigenomic defects in scaffold/matrix attachment regions (S/MAR), as
262 some correlate with origins of replication [30]. This phenotype could be exacerbated by
263 multiple mitotic divisions of follicular epithelial cells during oogenesis, and/or the
264 presence of holocentric chromosomes [31]. Future work describing the epigenomic
265 contributions to cellular defects are now possible to study in this newly emerging and
266 tractable model species, *O. fasciatus*.

267 We suggest that DNA methylation is more important for genome structure,
268 integrity or other cellular processes than it is for somatic expression in *O. fasciatus*.
269 Although it is possible that DNA methylation is important for expression control in a rare
270 cell type that was not examined in our study, we instead propose that regulation of
271 expression is not likely the primary role in insects. Investigating the importance of DNA

methylation prior to the onset of meiosis and throughout embryo development is a fundamental next-step. *Oncopeltus fasciatus* represents a fruitful model species for functional studies of DNA methylation, and continuation of studies in this system will unravel the insect epigenome and its functional consequences.

Materials and Methods

Phylogenetic analysis. A subset of DNA methyltransferase (*Dnmt*) 1, 2, and 3 sequences was obtained from [16] for phylogenetic analysis. The subset included only insect species with available MethylC-seq data, and species representatives from the dipteran suborders Brachycera and Nematocera: *Acyrthosiphon pisum*, *Aedes aegypti*, *Aedes albopictus*, *Anopheles gambiae*, *Apis mellifera*, *Bombyx mori*, *Camponotus floridanus*, *Copidosoma floridanum*, *Culex pipiens quinquefasciatus*, *Drosophila melanogaster*, *Harpegnathos saltator*, *Microplitis demolitor*, *Nasonia vitripennis*, *Nicrophorus vespilloides*, *O. fasciatus*, *Ooceraea* (*Cerapachys*) *biroi*, *Polistes canadensis*, *Polistes dominula*, *Solenopsis invicta*, *Tribolium castaneum*, and *Zootermopsis nevadensis*. DNA methyltransferases were reassessed in *O. fasciatus* by using InterProScan v5.23-62.0 [32] to identify annotated proteins with a C-5 cytosine-specific DNA methylase domain (PF00145). Sequence identifiers are located in S1 Fig. Full-length protein sequences were aligned using PASTA v1.6.4 [33], and manually trimmed of divergent, non-homologous sequence in Mesquite v3.2 [34]. Full, aligned, and aligned and trimmed sequence alignments, and parenthetical phylogenetic tree are located in S1 Data. Phylogenetic relationship among *Dnmt* sequences was estimated using BEAST v2.3.2 [35] with a

295 Blosum62+Γ model of amino acid substitution. A Markov Chain Monte Carlo (MCMC)
296 was ran until stationarity and convergence was reached (10,000,000 iterations), and a
297 burnin of 1,000,000 was used prior to summarizing the posterior distribution of tree
298 topologies. A consensus tree was generated using TreeAnnotator v2.3.2, visualized in
299 FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and exported for stylization in
300 Affinity Designer v1.5.1 (https://affinity.serif.com/en-us/).
301
302 **PCR confirmation for the presence of a single *Dnmt1* ortholog in *O. fasciatus*.** To
303 determine if OFAS015351 and OFAS018396 were two parts of a single *Dnmt1* ortholog,
304 we designed one sense primer at the 3' end of OFAS015351 (Of_DMNT1-1_3603S; S1
305 Table) and two antisense primers at the 3' end of OFAS018396 (Of_DNMT1-2_424A
306 and Of_DNMT1-2_465A; S1 Table). A fourth sense primer (Of_DNMT1-2_1S; S1
307 Table) was designed at the 5' end of OFAS018396 to confirm the size and sequence of
308 this possibly truncated gene annotation.
309 Polymerase Chain Reaction (PCR) with primer combinations Of_DMNT1-
310 1_3603S–Of_DNMT1-2_424A, Of_DMNT1-1_3603S–Of_DNMT1-2_465A, and
311 Of_DNMT1-2_1S–Of_DNMT1-2_424A was performed using Q5 Polymerase (New
312 England BioLabs, Ipswich, MA) per manufactures instructions. Thermacycler conditions
313 were 98°C for 15 seconds (s) (denaturing), 60°C for 30 s (annealing), and 72°C for 30 s
314 (extension), and repeated for 40 cycles. The PCR products were then purified using
315 QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands), and sequenced at the
316 Georgia Genomics and Bioinformatics Core (Athens, GA).
317

318 **Animal culture.** *Oncopeltus fasciatus* cultures were originally purchased from Carolina
319 Biologicals (Burlington, NC). Mass colonies were maintained in incubators under a 12
320 h:12 h light/dark cycle at 27°C. Colonies and individual experimental animals were fed
321 organic raw sunflower seeds and provided with *ad libitum* deionized water. Late instar
322 nymphs were separated from the mass colonies and housed under the same conditions.
323 Nymph colonies were checked daily for newly emerged adults. Adults were separated by
324 sex and kept with food and water for 7–10 days until females reached sexual maturity.
325
326 **Parental RNAi.** Template for the *in vitro* transcription of reactions was prepared from a
327 PCR reaction in which T7 phage promoter sequences were added to the gene-specific
328 *Dnmt1* primers [36]. For our control sequence, we used the red fluorescent protein (*Red*)
329 sequence used in previous parental RNAi experiments in *O. fasciatus* [36] or buffer.
330 Primer sequences can be found in S2 Table. Sense and anti-sense RNA was synthesized
331 in a single reaction using the Ambion MEGAscript kit (ThermoFisher Sci, Waltham,
332 MA). After purification, the double-stranded RNA (dsRNA) concentration was adjusted
333 to 2 μg/μL in injection buffer (5 mM KCl, 0.1 mM NaH_2PO_4) [36]. Females were
334 injected with 5 μL of dsRNA between the abdominal sternites using an insulin syringe.
335 Following injection, females were paired with an un-injected male to stimulate
336 oviposition and fertilize eggs. Individual females with their mate were housed in petri
337 dishes with sunflower seeds, water and cotton wool as an oviposition site. The parental
338 RNAi protocol has been reported to result in 100% penetrance by the third clutch of eggs
339 [36] and this was also our experience.
340

Reproductive phenotype screening and analysis. Eggs were collected between days 4–10 post-injection and assessed for development. *Oncopeltus fasciatus* embryos change from a creamy white color to orange as they develop, which indicates viability. Thus, color change is a useful tool for assessing healthy development. We examined the number of developing eggs at 5 days post-oviposition, at which point viable eggs are clearly distinguishable from inviable eggs, as well as hatching rate of eggs from females injected with double-stranded *Dnmt1* (ds-*dnmt1*) and double-stranded *Red* (ds-*red*). The data for the number of eggs laid was normally distributed so differences among RNAi treated groups were tested using analysis of variance (ANOVA). The development data, however, was not normally distributed and consisted of binary states (developed and not developed), and so differences among treatment groups were analyzed with a Generalized Linear Model (GLM) using a Poisson distribution.

A second set of *O. fasciatus* females were injected in the same manner as described in **Parental RNAi** to assess post-transcriptional knockdown of *Dnmt1* on ovarian structure. *Oncopeltus fasciatus* females were dissected 10 days after injection. By 10 days post-injection *O. fasciatus* females are beginning to stop laying recognizable eggs. Ovaries were removed from *O. fasciatus* females and placed in 1× PBS. Whole ovaries were imaged with a Leica DFC295 stereomicroscope using Leica Application Suite morphometric software (LAS V4.1; Leica, Wetzlar, Germany). Dissected ovaries were fixed within 15 minutes of dissection in 4% formaldehyde in 1× PBS for 25 minutes. Fixed ovaries were stained with Hoechst 33342 (Sigma Aldrich) at 0.5 µg/ml. The stained ovarioles were imaged using a Zeiss LSM 710 Confocal Microscope (Zeiss) at the University of Georgia Biomedical Microscopy Core.

364 **Quantitative RT-PCR.** To assess the effectiveness of post-transcriptional knockdown of
365 *Dnmt1*, females were dissected 11 days post-injection. Ovaries were removed from each
366 female, flash frozen in liquid nitrogen and stored at -80°C until processing. Total RNA
367 (and DNA) was extracted from a single ovary per female using a Qiagen Allprep
368 DNA/RNA Mini Kit (Qiagen, Venlo, The Netherlands) per manufacturer's instructions.
369 Complementary DNA (cDNA) was synthesized from 500 ng RNA with qScript cDNA
370 SuperMix (Quanta Biosciences, Gaithersburg, MD).

371 Expression level of *Dnmt1* was quantified by quantitative real-time PCR (qRT-
372 PCR). Primers were designed for *Dnmt1* using the *O. fasciatus* genome as a reference
373 [37]. Actin and GAPDH were used as endogenous reference genes. Primer sequences can
374 be found in Extended Data Table 2. We used Roche LightCycler 480 SYBR Green
375 Master Mix with a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN) for
376 qRT-PCR. All samples were run with 3 technical replicates using 10 μL reactions using
377 the manufacturer's recommended protocol. Primer efficiency calculations, genomic
378 contamination testing and endogenous control gene selection were performed as
379 described by [37]. We used the ΔΔCT method [38] to examine differences in expression
380 between control and ds-*dnmt1* injected females [37].

381

382 **Whole-Genome Bisulfite Sequencing (WGBS) and analysis of cytosine (DNA)**
383 **methylation.** MethylC-seq libraries for an *O. fasciatus* ds-*dnmt1* and control individual
384 were prepared according to the protocol described in [39] using genomic DNA extracted
385 from ovaries (see **Materials and Methods** section **Quantitative RT-PCR**). Libraries
386 were single-end 75 bp sequenced on an Illumina NextSeq500 machine [40, 41].

Unmethylated lambda phage DNA was used to as a control for sodium bisulfite conversion, and an error rate of ~0.05% was estimated. *Oncopeltus fasciatus* ds-*dnmt1* and control were sequenced to a depth of ~18× and ~21×, which corresponded to an actual mapped coverage of ~9× and ~11×, respectively. Additionally, low pass (< 1×) WGBS from gut, head, ovary, and thorax was performed for three control and ds-*dnmt1* biological replicates. *Blattella germanica* was additionally sequenced to generate equal numbers of hemi– and holometabolous insects investigated in this study. However, DNA was extracted from whole-body minus gastrointestinal tract. MethylC-seq libraries were prepared and sequenced identically to *O. fasciatus*. An error rate of ~0.14% was estimated from unmethylated lambda phage DNA. *Blattella germanica* was sequenced to a depth of ~8×, which corresponded to an actual mapped coverage of ~5×. WGBS data for *O. fasciatus* and *B. germanica* can be found on Gene Expression Omnibus (GEO) under accession GSE109199. Previously published WGBS data for *A. mellifera* [42], *Bo. mori* [17], *Daphnia pulex* [43], *N. vespilloides* [19], and *Z. nevadensis* [15] were downloaded from the Short Read Archive (SRA) using accessions SRR445803–4, SRR027157–9, SRR1552830, SRR2017555, and SRR3139749, respectively. Thus, DNA methylation was investigated for six insects from six different orders spread evenly across developmental groups, and a crustacean outgroup. WGBS data was aligned to each species respective genome assembly using the methylpy pipeline [44]. In brief, reads were trimmed of sequencing adapters using Cutadapt v1.9 [45], and then mapped to both a converted forward strand (cytosines to thymines) and converted reverse strand (guanines to adenines) using bowtie v1.1.1 [46]. Reads that mapped to multiple locations, and clonal reads were removed.

Weighted DNA methylation was calculated for CG sites by dividing the total number of aligned methylated reads by the total number of methylated plus unmethylated reads [47]. For genic metaplots, the gene body (start to stop codon), 1000 base pairs (bp) upstream, and 1000 bp downstream was divided into 20 windows proportional windows based on sequence length (bp). Weighted DNA methylation was calculated for each window and then plotted in R v3.2.4 (https://www.r-project.org/). CG sequence context enrichment for each gene was determined through a binomial test followed by Benjamini-Hochberg false discovery rate [48, 49]. A background mCG level was determined from all coding sequence, which was used as a threshold in determining significance with a False Discovery Rate (FDR) correction. Genes were classified as CG-methylated if they had reads mapping to at least 20 reads mapping to 20 CG sites and a $q < 0.05$. Using a binomial test can lead to false-negatives – highly CG-methylated genes that are classified as unmethylated (UM) – due to a low number of statistically CG-methylated sites (S4 Table). Genes classified as unmethylated, but had a mCG level greater than the lowest CG-methylated gene were dropped from future analyses.

Ortholog identification. Best BLASTp hit (arguments: -max_hsps 1 –max_target_seqs 1 evalue 1e-03) was used to identify orthologs between *O. fasciatus* and other insect species investigated.

RNA-seq and differential expression analysis. RNA-seq libraries for RNA extracted from ovaries of three biological *O. fasciatus* ds-*dnmt1* and control replicates at 11 days post-injection were constructed using Illumina TruSeq Stranded RNA LT Kit (Illumina,

San Diego, CA) following the manufacturer's instructions with limited modifications. RNA from ovaries of an additional three biological *O. fasciatus* ds-*dnmt1* and control replicates, and three biological *O. fasciatus* ds-*dnmt1* and control replicates from gut, head, and thorax were extracted. The starting quantity of total RNA was adjusted to 1.3 µg, and all volumes were reduced to a third of the described quantity. Libraries were single-end 75 bp sequenced on an Illumina NextSeq500 machine. RNA-seq data for *O. fasciatus* ds-*dnmt1* and control can be found on GEO under accession GSE109199. Previously published RNA-seq data for *A. mellifera* [50], and *Z. nevadensis* (15) were downloaded from the SRA using accessions SRR2954345, and SRR3139740, respectively.

Raw RNA-seq FASTQ reads were trimmed for adapters and preprocessed to remove low-quality reads using Trimmomatic v0.33 (arguments: LEADING:10 TRAILING:10 MINLEN:30) [51] prior to mapping to the *O. fasciatus* v1.1 reference genome assembly. Reads were mapped using TopHat v2.1.1 [52] supplied with a reference General Features File (GFF) to the *O. fasciatus* v1.1 reference genome assembly [26], and with the following arguments: -I 20000 --library-type fr-firststrand --b2-very-sensitive.

Differentially expressed genes (DEGs) between ds-*dnmt1* and control libraries were determined using edgeR v3.20.1 [53] implemented in R v3.2.4 (https://www.r-project.org/). Genes were retained for DEG analysis if they possessed a Counts Per Million (CPM) $\geq$ 1 in at least $\geq$ 2 libraries. Significance was determined using the glmQLFTest function, which uses empirical Bayes quasi-likelihood F-tests. Parameter settings were determined following best practices for DEG analysis as described by [54].

Gene expression metrics for *A. mellifera*, *Z. nevadensis*, and *O. fasciatus* ds-*dnmt1* and control are located in S5–8 Tables, respectively.

Gene Ontology (GO) annotation and enrichment. GO terms were assigned to *O. fasciatus* v1.1 gene set [26] through combining annotations from Blast2Go PRO v4.1.9 [55], InterProScan v5.23-62.0 (arguments: -goterms -iprlookup -appl CDD,Pfam) [32], and through sequence homology to *D. melanogaster* using BLASTp (arguments: -evalue 1.0e-03 -max_target_seqs 1 -max_hsps 1). 11,105/19,615 gene models were associated with at least one GO term and a total of 8,190 distinct GO identifiers were mapped. GO terms are found in S11 Table. Enriched GO terms in gene groups were evaluated using topGO v2.30.0 [56] implemented in R v3.2.4 (https://www.r-project.org/), and significance ($p < 0.05$) of terms was assessed using Fisher's exact test with a weighted algorithm. Gene groups were contrasted to all *O. fasciatus* genes associated with GO terms (S12 Table).

Transposable element (TE) annotation and expression. TEs were identified using RepeatMasker v4.0.5 (http://www.repeatmasker.org) provided with the invertebrate repeat library from Repbase (http://www.girinst.org/repbase/) (arguments: -lib <Repbase invertebrate library> -no_is -engine wublast -a -inv -x -gff. Following RepeatMasker, neighboring TEs of the same type were collapsed into a single locus within the outputted GFF. The unmodified GFF is located in S9 Table.

To quantify expression from TEs RNA-seq libraries from *O. fasciatus* ds-*dnmt1* and control were independently combined and mapped to the *O. fasciatus* v1.1 reference

479 genome assembly [26] using bowtie2 v2.2.9 [57] with the following arguments: --
480 sensitive. Mapped reads overlapping with the top ten most abundant TEs of ≥ 500 bp in
481 length were identified using the *intersect* command in BEDTools suite v 2.26.0 [58]. TE
482 expression is quantified as Reads Per Kilobase per Million mapped reads (RPKM) for
483 each intersected TE type by counting the number reads and dividing by the mapped
484 library read number in millions. Significance in expression of TEs between ds-*dnmt1* and
485 control tissues was assessed using the Mann-Whitney test with the alternative hypothesis
486 set to "greater" in R v3.2.4 (https://www.r-project.org/).
487
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References

1. Falckenhayn C, Boerjan B, Raddatz G, Frohme M, Schoofs L, Lyko F. Characterization of genome methylation patterns in the desert locust *Schistocerca gregaria*. J Exp Biol. 2013;216: 1423–1429.
2. Cullen DA, Cease AJ, Latchininsky AV, Ayali A, Berry K, Buhl J, et al. From molecules to management: mechanisms and consequences of locust phase polyphenism. Adv In Insect Phys. 2017;53: 167–285.
3. Pegoraro M, Bafna A, Davies NJ, Shuker DM, Tauber E. DNA methylation changes induced by long and short photoperiods in *Nasonia*. Genome Res. 2016;26: 203–210.
4. Reynolds JA. Epigenetic influences on diapause. Adv In Insect Phys. 2017;53: 115–144.
5. Kozeretska IA, Serga SV, Koliada AK, Vaiserman AM. Epigenetic regulation of longevity in insects. Adv In Insect Phys. 2017;53: 87–114.
6. Bonasio R, Li Q, Lian J, Mutti NS, Jin L, Zhao H, et al. Genome-wide and caste-specific DNA methylomes of the ants *Camponotus floridanus* and *Harpegnathos saltator*. Curr Biol. 2012;22: 1755–1764.
7. Foret S, Kucharski R, Pellegrini M, Feng S, Jacobsen SE, Robinson GE, et al. DNA methylation dynamics, metabolic fluxes, gene splicing, and alternative phenotypes in honey bees. Proc Natl Acad Sci USA. 2012;109: 4968–4973.
8. Lockett GA, Kucharski R, Maleszka R. DNA methylation changes elicited by social stimuli in the brains of worker honey bees. Genes Brain Behav. 2012;11: 235–242.

9. Ford D. Honeybees and cell lines as models of DNA methylation and aging in response to diet. Exp Gerontol. 2013;48: 614–619.
10. He XJ, Zhou LB, Pan QZ, Barron AB, Yan WY, Zeng ZJ. Making a queen: an epigenetic analysis of the robustness of the honeybee (*Apis mellifera*) queen developmental pathway. Mol Ecol. 2017;26: 1598–1607.
11. Libbrecht R, Oxley PR, Keller L, Kronauer DJ. Robust DNA methylation in the clonal raider ant brain. Curr Biol. 2016;26: 391–395.
12. Bewick AJ, Vogel KJ, Moore AJ, Schmitz RJ. Evolution of DNA methylation across insects. Mol Biol Evol. 2016;34: 654–665.
13. Glastad KM, Arsenault SV, Vertacnik KL, Geib SM, Kay S, Danforth BN, et al. Variation in DNA methylation is not consistently reflected by sociality in Hymenoptera. Genome Biol Evol. 2017;9: 1687–1698.
14. Provataris P, Meusemann K, Niehuis O, Grath S, Misof M. Signatures of DNA methylation across insects suggest reduced DNA methylation levels in Holometabola. Genome Biol Evol. 2018;10: 1185–1197.
15. Glastad KM, Gokhale K, Liebig J, Goodisman MAD. The caste- and sex-specific DNA methylome of the termite *Zootermopsis nevadensis*. Sci Rep. 2016;6: 37110.
16. Lyko F, Foret S, Kucharski R, Wolf S, Falckenhayn C, Maleszka R. The honey bee epigenomes: differential methylation of brain DNA in queens and workers. PLoS Biol. 2010;8 :e1000506.
17. Xiang H, Zhu J, Chen Q, Dai F, Li X, Li M, et al. Single base-resolution methylome of the silkworm reveals a sparse epigenomic map. Nat Biotechnol. 2010;28: 516–520.

546 18. Wang X, Wheeler D, Avery A, Rago A, Choi JH, Colbourne JK, et al. Function and
547 evolution of DNA methylation in *Nasonia vitripennis*. PLoS Genet. 2013;9:
548 e1003872.
549 19. Cunningham CB, Ji L, Wiberg RA, Shelton J, McKinney EC, Parker DJ, et al. The
550 genome and methylome of a beetle with complex social behavior, *Nicrophorus*
551 *vespilloides* (Coleoptera: Silphidae). Genome Biol Evol. 2015;7: 3383–3396.
552 20. Patalano S, Vlasova A, Wyatt C, Ewels P, Camara F, Ferreira PG, et al. Molecular
553 signatures of plastic phenotypes in two eusocial insect species with simple societies.
554 Proc Natl Acad Sci USA. 2015;112: 13970–13975.
555 21. Rehan SM, Glastad KM, Lawson SP, Hunt BG. The genome and methylome of a
556 subsocial small carpenter bee, *Ceratina calcarata*. Genome Biol Evol. 2016;8: 1401–
557 1410.
558 22. Sarda S, Zeng J, Hunt BG, Yi SV. The evolution of invertebrate gene body
559 methylation. Mol Biol Evol. 2012;29: 1907–1916.
560 23. Hunt BG, Glastad KM, Yi SV, Goodisman MAD. Patterning and regulatory
561 associations of DNA methylation are mirrored by histone modifications in insects.
562 Genome Biol Evol. 2013;5: 591–598.
563 24. Li-Byarlay H, Li Y, Stroud H, Feng S, Newman TC, Kaneda M, et al. RNA
564 interference knockdown of DNA methyl-transferase 3 affects gene alternative
565 splicing in the honey bee. Proc Natl Acad Sci USA. 2013;110: 12750–12755.
566 25. Chipman AD. *Oncopeltus fasciatus* as an evo-devo research organism. Genesis.
567 2017;**55**.

568 26. Panfilio KA, Vargas Jentzsch IM, Benoit JB, Erezyilmaz D, Suzuki Y, Colella S, et
569 al. Molecular evolutionary trends and feeding ecology diversification in the
570 Hemiptera, anchored by the milkweed bug genome; 2017. Preprint. Available from:
571 doi:https://doi.org/10.1101/201731. Cited 26 June 2018.
572 27. Liu PZ, Kaufman TC. Hunchback is required for suppression of abdominal identity,
573 and for proper germband growth and segmentation in the intermediate germband
574 insect *Oncopeltus fasciatus*. Development. 2004;131: 1515–1527.
575 28. Bewick AJ, Hofmeister BT, Lee K, Zhang X, Hall DW, Schmitz RJ. FASTmC: A
576 suite of predictive models for nonreference-based estimations of DNA methylation.
577 G3 (Bethesda). 2015;6: 447–752.
578 29. Bonhag PF, Wick JR. The functional anatomy of the male and female reproductive
579 systems of the milkweed bug, *Oncopeltus fasciatus* (Dallas) (Heteroptera:
580 Lygaeidae). J Morphol. 1953;93: 177–283.
581 30. Vaughn JP, Dijkwel PA, Mullenders LH, Hamlin JL. Replication forks are associated
582 with the nuclear matrix. Nucleic Acids Res. 1990;18: 1965–1969.
583 31. Drinnenberg IA, deYoung D, Henikoff S, Malik HS. Recurrent loss of CenH3 is
584 associated with independent transitions to holocentricity in insects. eLife. 2014;doi:
585 10.7554/eLife.03676.
586 32. Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, et al. InterProScan 5:
587 genome-scale protein function classification. Bioinformatics. 2014;30: 1236–1240.
588 33. Mirarab S, Nguyen N, Guo S, Wang LS, Kim J, Warnow T. PASTA: ultra-large
589 multiple sequence alignment for nucleotide and amino-acid sequences. J Comput
590 Biol. 2015;22: 377–386.

591 34. Maddison WP, Maddison DR. Mesquite: a modular system for evolutionary analysis.
592 Version 3.31. 2017;http://mesquiteproject.org.
593 35. Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu CH, Xie D. et al. BEAST 2: a
594 software platform for Bayesian evolutionary analysis. PLoS Comput. Biol. 2014;**10**:
595 e1003537.
596 36. Ewen-Campen B, Jones TE, Extavour CG. Evidence against a germplasm in the
597 milkweed bug *Oncopeltus fasciatus*, a hemimetabolous insect. Biol Open. 2013;2:
598 556–568.
599 37. Cunningham CB, Douthit MK, Moore AJ. Octopaminergic gene expression and
600 flexible social behaviour in the subsocial burying beetle *Nicrophorus vespilloides*.
601 Insect Mol Biol. 2014;23: 391–404.
602 38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
603 quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25: 402–408.
604 39. Urich MA, Nery JR, Lister R, Schmitz RJ, Ecker JR. MethylC-seq library preparation
605 for base-resolution whole-genome bisulfite sequencing. Nat Protoc. 2015;10: 475–
606 483.
607 40. Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, et al. Shotgun
608 bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation
609 patterning. Nature. 2008;452: 215–219.
610 41. Lister R, O'Malley RC, Tonti-Filippini J, Gregory BD, Berry CC, Millar AH, et al.
611 Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. Cell.
612 2008;133: 523–536.

613 42. Herb BR, Wolschin F, Hansen KD, Aryee MJ, Langmead B, Irizarry R, et al.
614 Reversible switching between epigenetic states in honeybee behavioral subcastes. Nat
615 Neurosci. 2012;15: 1371–1373.
616 43. Asselman J, De Coninck DIM, Pfrender ME, De Schamphelaere KAC. Gene body
617 methylation patterns in *Daphnia* are associated with gene family size. Genome Biol
618 Evol. 2016;8: 1185–1196.
619 44. Schultz MD, He Y, Whitaker JW, Hariharan M, Mukamel EA, Leung D, et al.
620 Human body epigenome maps reveal noncanonical DNA methylation variation.
621 Nature. 2015;523: 212–216.
622 45. Martin M, Marcel M. Cutadapt removes adapter sequences from high-throughput
623 sequencing reads. EMBnet J. 2011;17: 10–12.
624 46. Langmead B, Trapnell C, Salzberg SL. Ultrafast and memory-efficient alignment of
625 short DNA sequences to the human genome. Genome Biol. 2009;10: R25.
626 47. Schultz MD, Schmitz RJ, Ecker JR. 'Leveling' the playing field for analyses of single-
627 base resolution DNA methylomes. Trends Genet. 2012;28: 583–585.
628 48. Takuno S, Gaut BS. Body-methylated genes in *Arabidopsis thaliana* are functionally
629 important and evolve slowly. Mol Biol Evol. 2012;1: 219–227.
630 49. Niederhuth CE, Bewick AJ, Ji L, Alabady MS, Kim KD, Li Q, et al. Widespread
631 natural variation of DNA methylation within angiosperms. Genome Biol. 2016;17:
632 194.
633 50. Vleurinck C, Raub S, Sturgill D, Oliver B, Beye M. Linking genes and brain
634 development of honeybee workers: a whole-transcriptome approach. PLoS One.
635 2016;11: e0157980.

636 51. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina
637 sequence data. Bioinformatics. 2014;30: 2114–2120.
638 52. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate
639 alignment of transcriptomes in the presence of insertions, deletions and gene fusions.
640 Genome Biol. 2013;14: R36.
641 53. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for
642 ifferential expression analysis of digital gene expression data. Bioinformatics.
643 2010;26: 139–140.
644 54. Chen Y, Lun ATL, Smyth GK. From reads to genes to pathways: differential
645 expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-
646 likelihood pipeline. F1000Res. 2016;5: 1438.
647 55. Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, et al.
648 High-throughput functional annotation and data mining with the Blast2GO suite.
649 Nucleic Acids Res. 2008;36: 3420–3435.
650 56. Alexa A, Rahnenfuhrer J. topGO: Enrichment analysis for gene ontology. R package
651 version 2.30.0. 2016.
652 57. Langmead B, Salzberg S. Fast gapped-read alignment with Bowtie 2. Nat Methods.
653 2012;9: 357–359.
654 58. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic
655 features. Bioinformatics. 2010;26: 841–842.

Supporting Information

S1 Fig. Identification of *O. fasciatus* DNA methyltransferases. (A) Phylogenetic relationship of DNA methyltransferases identified the *de novo* (*Dnmt3*) and maintenance (*Dnmt1*) DNA methyltransferase in *O. fasciatus*. Node support with ≤0.5 posterior probability is indicated – other nodes are ≥0.95. Branch lengths are in amino acid substitutions per site. Species names are represented as abbreviations: Acy. pis.: *Acyrthosiphon pisum*, Aed. aeg.: *Aedes aegypti*, Aed. alb.: *Aedes albopictus*, Ano. gam.: *Anopheles gambiae*, Api. mel.: *A. mellifera*, Bom. mor.: *Bo. mori*, Cam. flo.: *Camponotus floridanus*, Cop. flo.: *Copidosoma floridanum*, Cul. qui.: *Culex pipiens quinquefasciatus*, Dro. mel.: *Drosophila melanogaster*, Har. sal.: *Harpegnathos saltator*, Mic. dem.: *Microplitis demolitor*, Nas. vit.: *Nasonia vitripennis*, Nic. ves.: *Nicrophorus vespilloides*, Onc. fas.: *O. fasciatus*, Cer. bir.: *Ooceraea* (*Cerapachys*) *biroi*, Pol. can.: *Polistes canadensis*, Pol. dom.: *Polistes dominula*, Sol. inv.: *Solenopsis invicta*, Tri. cas.: *Tribolium castaneum*, and Zoo. nev.: *Z. nevadensis*. (B) A to scale representation of *Dnmt1* and protein domains identified in *O. fasciatus* and *M. musculus*.

S2 Fig. DNA methylation consequences following post-transcriptional knockdown of *Dnmt1* are restricted to ovaries. (A) Assessment of RNAi treatment targeting *Dnmt1* using qRT-PCR demonstrates successful reduction in transcription compared to control across all tissues sampled. Colored dots indicate independent biological replicates. (B) Genome-wide CG methylation level across tissues sampled. Numbers at the top of each bar corresponds to independent biological replicates.

679

680 **S3 Fig. Eggs laid in *O. fasciatus* ds-*dnmt1* and control females.** (A) Number of eggs
681 laid by ds-*dnmt1* and control females 8-days post-injection. Dots indicate mean
682 expression level, and error bars indicate standard error of the mean.

683

684 **S4 Fig. mCG in *A. mellifera* and *Z. nevadensis* is not associated with transcription.**
685 (A) Gene expression level for deciles of increasing mCG (1–10) and un-methylated genes
686 (UM). Error bars represent 95% confidence interval of the mean. (B) Regression of gene
687 expression against a continuous measure of mCG among all genes with >0 FPKM and
688 weighted mCG. Raw *p* values are provided for each regression, and significance (S) or
689 non-significance (NS) is indicated in brackets following Bonferroni correction.

690

691 **S5 Fig. Loss of mCG in *O. fasciatus* ovaries has a limited effect on transcription.** (A)
692 Combinational overlap of genes that are differential CG-methylated and expressed, and
693 similarly CG-methylated and expressed between *O. fasciatus* ds-*dnmt1* and control. Gene
694 groups: Differentially Methylated Gene (DMG), Differentially Expressed Gene (DEG),
695 Similarly Methylated Gene (SMG)/UnMethylated Genes (UMG), and non-Differentially
696 Expressed Gene (non-DEG). More stringent thresholds were used to group genes as CG-
697 methylated or unmethylated (see **Materials and Methods**).

698

699 **S6 Fig. DNA methylation of TEs in *O. fasciatus*.** (A) Levels of mCG across the bodies
700 and 1kb flanking sequence of different annotated TEs in *O. fasciatus*.

701

S1 Table. PCR primers used to validate the presence of a single *Dnmt1* ortholog in the *O. fasciatus* genome.

S2 Table. Primer sequences for use in producing template DNA for use in the MegaScript transcription kit to generate double stranded RNAs for injection and for quantitative real-time PCR to assess expression levels of *Dnmt1*.

S3 Table. Overlap between *O. fasciatus* (control) CG-methylated and unmethylated genes in none and ≥ 1 other insect species used in this study.

S4 Table. DNA methylation summary statistics for all species investigated in this study.

S5 Table. Output from edgeR v3.20.1 [53] with gene expression (Fragments Per Kilobase of transcript per Million mapped reads [FPKM]) for *O. fasciatus* ovaries ds-*dnmt1* and control biological and technical replicates.

S6 Table. Gene expression FPKM for *A. mellifera* queen and drone brains.

S7 Table. Gene expression (FPKM) for *Z. nevadensis* female worker at the final instar larva.

724 **S8 Table. Output from edgeR v3.20.1 [53] with gene expression (FPKM) for *O.***
725 ***fasciatus* gut, head, and thorax ds-*dnmt1* and control biological replicates.**
726
727 **S9 Table. Output General Features File (GFF) from RepeatMasker v4.0.5**
728 **(http://www.repeatmasker.org).**
729
730 **S10 Table. Significance (*p* value) of TE expression between ds-*dnmt1* and control**
731 **tissues.**
732
733 **S11 Table. Gene Ontology (GO) terms for *O. fasciatus* v1.1 reference genome**
734 **assembly [26] annotated genes.**
735
736 **S12 Table. Significantly enriched GO terms for the intersections between**
737 **Differentially Methylated Genes (DMG), Similarly Methylated Genes (SMG),**
738 **Differentially Expressed Genes (DEG), and non-Differentially Expressed Genes**
739 **(non-DEG).**
740
741 **S1 Data. Unaligned and aligned DNA methyltransferase protein sequences in fasta**
742 **format, and a parenthetical phylogenetic tree in nexus format estimated from the**
743 **aligned DNA methyltransferase protein sequences using BEAST v2.3.2 [35].**