Running title: ***Dmnt1* and *Wnt* in the production of oocytes**

An association between *Dmnt1* and *Wnt* in the production of oocytes in the whitefly *Bemisia tabaci*

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Abstract

Producing viable gametes among metazoans relies on generally conserved genetic machinery. We have previously shown that *DNA methyltransferase I* (*Dnmt1*) plays an important role for both male and female gametogenesis in insects. In this study, we tested the hypothesis that *Dmnt1* influences meiosis, a prediction from previous studies that suggested this is where gametogenesis is being perturbed. We used RNAi to reduce the gene expression of *Dnmt1* within the whitefly *Bemisia tabaci*, a Hemipteran species that relies on *Dnmt1* for proper gametogenesis. We then used RNA-seq to test an *a priori* hypothesis that meiosis related molecular pathways would be perturbed. This was done by comparing *Dnmt1* RNAi and control samples. We generally did not find an overall effect on meiosis genes. However, we found that genes in the *Wnt* pathway, genes associated with the entry into meiosis in vertebrates, were differentially expressed when *Dnmt1* expression was experimentally reduced. Our results are consistent with *Dnmt1* knockdown influencing specific pathways and not causing general transcriptional response. This is a finding that is also seen with other insect species. We also characterized the methylome of *B. tabaci* and assessed the influence of *Dnmt1* knockdown on cytosine methylation. This species has methylome characteristics comparable to other Hemipterans regarding overall level, enrichment within gene bodies, and bimodal distribution of methylated/non-methylated genes. Very little differential methylation was observed, and difference of methylation were not associated with differences of gene expression. The effect on *Wnt* presents an interesting new candidate pathway for future studies.

KEYWORDS

DNA methylation, epigenetics, gametogenesis, reproduction

Introduction

Despite the variation of *DNA methyltransferase 1* (*Dnmt1*) copy numbers across Insecta, from completely lacking to the presence of multiple functional copies (Bewick et al, 2017), one common feature is its role during insect gametogenesis. Reproduction relies on producing viable gametes as a starting point. This process at a molecular level is generally conserved among metazoans, but variation among lineages does exist (Ewen-Campen et al., 2010; Whittle & Extravour, 2019). The canonical function of *Dnmt1* is replicating cytosine methylation pattern on newly synthesized strands of DNA after duplication during cell division (Law & Jacobsen, 2010). It is a highly conserved gene across the tree of life and performs this function for insects (Schmitz et al., 2019; Gladstad et al., 2019; Duncan et al., 2022). This gene also has a role during gametogenesis of many male and female insects (Zwier et al., 2012; Schulz et al., 2018; Bewick et al., 2019; Li et al. 2019; Amukamara et al., 2020; Gegner et al., 2020; Ventós-Alfonso et al., 2020; Takada et al., 2021; Washington et al., 2021; Arsala et al., 2022; Xu et al. 2022; Cunningham et al., 2023; Ivasky et al., 2023; Shelby et al. 2023). However, an increasing body of evidence suggests that this role is independent of its influence on DNA methylation patterns (Bewick et al., 2019; Amukamara et al., 2020; Washington et al., 2021; Cunningham et al., 2023; Ivasky et al., 2023). When *Dnmt1* gene expression is experimentally reduced, pre-meiotic germ cells arrest (Amukamara et al., 2020; Washington et al., 2021; Shelby et al., 2022; Cunningham et al., 2023; Ivasyk et al., 2023). Thus, understanding the role of *Dnmt1* during insect gametogenesis should further a foundational understanding of the molecular pathways of insect reproduction.

The whitefly *Bemisia tabaci* (Gennadius) is a global pest species (Stanley & Naranjo, 2012). As such, work has been done to understand its reproduction and the molecular pathways that underpin its reproduction with the intention to better control this pest (e.g., Tsueda and Tsuchida, 2011; Shelby et al., 2021; Hu et al., 2022; Shelby et al., 2023). In whiteflies, *Dnmt1* RNAi-treated females fail to produce as many eggs as control individuals and these eggs show reduced viability after oviposition (Shelby et al., 2023), which is consistent with other knockdown studies of *Dmnt1* (Bewick et al., 2019; Amukamara et al., 2020; Gegner et al., 2020; Washington et al., 2021; Cunningham et al., 2023; Ivasyk et al. 2023). The ovaries or gametes themselves do not show any gross signs of apoptosis, rather development of gametes alone seems perturbed. Given this germ cell-specific phenotype, a natural unknown to decipher is to determine the genetic mechanism(s) that underpin the deficits in gamete production and embryo viability for the whitefly. It is unclear if *Dnmt1* also acts through a cytosine methylation independent pathway for *B. tabaci* as it does for other insects (Bewick et al., 2019; Amukamara et al., 2020; Gegner et al., 2020; Washington et al., 2021; Cunningham et al., 2023; Ivasyk et al. 2023). DNMT1, the protein product of *Dnmt1*, was not found within the nucleus of maturing oocytes of *Dnmt1* knockdowns, so it is known that the egg and gamete deficit is produced in the absence of DNMT1 (Shelby et al., 2023). Also, among studies that assessed it, gametogenesis arrests at the mitosis-meiosis boundary as gametogonia transition to gametocytes (Amukamara et al., 2020; Washington et al., 2021; Cunningham et al., 2023; Ivasyk et al. 2023). This consistent phenotype suggests two possible mechanisms: (1) either meiosis is being inhibited or (2) germ cell maturation after meiosis is being inhibited. We believe that the preponderance of evidence suggests the former, but both remain formal possibilities (Amukamara et al., 2020; Takada et al., 2021; Washington et al., 2021; Cunningham et al., 2023; Ivasky et al., 2023).

The current study was designed to differentiate among the potential perturbations to gamete development caused by the knockdown of *Dnmt1* expression. *Dnmt1* knockdown in a related Hemipteran, *Oncopeltus fasciatus*, also results in loss of gametogenesis, but this effect is not underpinned by many

differentially expressed genes in either the ovary (Bewick et al., 2019) or the testes when the phenotype is first initiated (Cunningham et al., 2023). This led us to hypothesize that downregulation of *Dnmt1* is targeting specific genes or gene networks. First, we tested the hypothesis that *Dnmt1* gene expression reduction would be associated with perturbation of meiosis molecular pathways, but not mitotic or general cell cycling molecular pathways. This required us to contrast the influence of *Dmnt1* knockdown on each pathway. We did this in two ways. First, we used a panel of *a priori* candidate genes canonically associated with each process to contrast; general cell proliferation, cell cycling, mitosis, and meiosis. Second, we used Gene Ontology Terms representing these processes and assessed if they were enriched among the most differentially expressed gene between control and RNAi-treated samples. After this, we also assessed the general patterns of differential gene expression to explore the dataset for perturbed molecular pathways that can be hypotheses in the future. Our hypothesis that meiotic-related candidate genes would be preferentially perturbed was not strongly supported. Our second aim was to characterize the general patterns of cytosine methylation of this species, the effect of *Dnmt1* reduction on cytosine methylation, and the association between cytosine methylation and gene expression. *Bemisia tabaci* has cytosine methylation at a level comparable to other Hemiptera. However, reducing *Dnmt1* expression did not cause pervasive reduction of cytosine methylation using a global measure despite the severe reproductive deficit. From that a more detailed analysis remains to be done. The differences that are detectable are not associated with difference of gene expression. Future experiments assessing more tissue-specific level expression is now needed to determine how *Dmnt1* interacts with meiotic pathways.

Results

Dnmt1 RNAi knockdown

Consistent with Shelby and colleagues (2023), our RNAi treatment reduced the expression of *Dnmt1* (LOC109040312; $\log_2$ fold change: -0.0216, P = 0.0276).

a priori candidate gene screen

Our hypothesis that meiotic-specific candidate genes would be preferentially perturbed was not strongly supported; none of the 5 meiosis-specific candidate genes were differentially expressed (Table 1). Four of the 22 candidate genes were statistically differentially expressed after FDR thresholding (Table 1). These genes were *Wnt*-like genes, along with one *CDC* and one *SMC3*. All gene assessed both here and by Shelby et al. (2023) had the same directional change and rank order of effect sizes.

Table 1. *a priori* candidate genes tested for differences between Control and *Dnmt1* knockdown treatments. Expression value and fold changes are from DESeq2. Bolded values are statisitcally signigicat after adaptive linear two-step procedure (Benjamini et al., 2006). Gene nomeclature is from OrthoDB.

Gene ID	Accession	Pathway	Standardized Expression	Fold Change	P value	*a priori* Category
Wingless (*Wnt* ortholog)	LOC109044616	Cell fate & proliferation	7.641	-0.001	0.463	General Cell Proliferation: 2/10
Wnt-1 -like	LOC109042201	Cell fate & proliferation	10.265	-0.005	0.560	
Wnt-2	LOC109041803	Cell fate & proliferation	8.729	0.001	0.512	
Wnt-4 -like	LOC109044333	Cell fate & proliferation	**10.343**	**0.012**	**0.009**	
Wnt-6 -like	LOC109044640	Cell fate & proliferation	**8.916**	**0.005**	**0.015**	
Wnt-10a -like	LOC109044612	Cell fate & proliferation	7.642	0.001	0.424	
Frizzled-2 -like	LOC109035614	Cell fate & proliferation	8.536	-0.004	0.222	
Frizzled-4 -like	LOC109030198	Cell fate & proliferation	9.002	-0.004	0.222	
Frizzled-5 -like	LOC109039814	Cell fate & proliferation	12.458	-0.001	0.933	
CDC20	LOC109035963	Cell cycle control	**12.465**	**-0.012**	**0.034**	Cell Cycle: 1/3
CYCD2	LOC109039503	Cell cycle control - G1/S-specific	10.915	0.005	0.533	
CYCD3	LOC109034725	Cell cycle control - G1/S-specific	8.745	-0.001	0.594	
Vasa	LOC109042479	Germ cell Development	15.291	0.001	0.856	Mitosis Specific: 1/4
Vasa	LOC109030586	Germ cell Development	15.052	-0.009	0.172	
CYCB3	LOC109043986	Cell cycle control - G2/mitotic-specific	12.196	-0.003	0.494	
SMC3 -like	LOC109038740	Chromosomal Structure Maintenance	**11.516**	**-0.025**	**0.050**	
Pumillio	LOC109041486	Repressor of Mitosis	10973.763	0.004	0.425	Meiosis Specific: 0/9
Decapentaplegic	LOC109031148	Repressor of Meiosis	147.197	0.001	0.787	
Decapentaplegic-like	LOC109032526	Repressor of Meiosis	34.130	-0.002	0.393	
Glass bottom boat	LOC109036141	Repressor of Meiosis	2223.091	0.000	0.968	
Boule	LOC109034894	meiotic G2/M transition	11.156	0.002	0.233	
SPO11	LOC109044039	Meiotic Recombination	7.689	-0.001	0.545	
MSH5	LOC109042904	Meiotic Recombination	8.962	-0.002	0.618	
MND1	LOC109040052	Meiotic Recombination	9.436	-0.001	0.857	
HOP2	LOC109030819	Meiotic Recombination	9.824	0.001	0.856	

***a priori* GO term enrichment**

Our hypothesis that the meiosis pathway would be the most affected by *Dnmt1* knockdown was not strongly supported by our *a priori* GO term enrichment analysis. Our hypothesis would have been supported if the meiosis GO Term were enriched. However, five of the six *a priori* GO

terms were statistically significantly, but these were among the genes that showed the least differences of expression, the opposite pattern predicted (Table 2). Namely, they were among the least differentially expressed genes between the control and RNAi-treatments.

Table 2. *a priori* GO term enrichment. Loss of *Dnmt1* does not preferentially target our predcited pathways. NES = normalized enrichment score.

Pathway	GO Term	Number of Annotated Genes	NES	FDR-adjusted P-value
Cell Cycle	GO:0007049	384	-4.742	**< 0.001**
Gametogenesis	GO:0007276	681	-4.260	**< 0.001**
Mitotic Cell Cycle	GO:0000278	255	-4.315	**< 0.001**
Meiotic Cell Cycle	GO:0051321	144	-3.385	**< 0.001**
Oogenesis	GO:0048477	491	-3.644	**< 0.001**
Apoptotic Process	GO:0006915	61	0.676	8.605E-01

Differential gene expression

We assessed the impact of *Dnmt1* knockdown to identify genes that were differentially expressed. The individual samples of each treatment clustered together, but the treatments were differentiated using a PCA and not statistically significantly different as the 95% confidence intervals were highly overlapping (Fig. 1). There were 96 genes (49 up-regulated, 47 down-regulated) between the treatments.

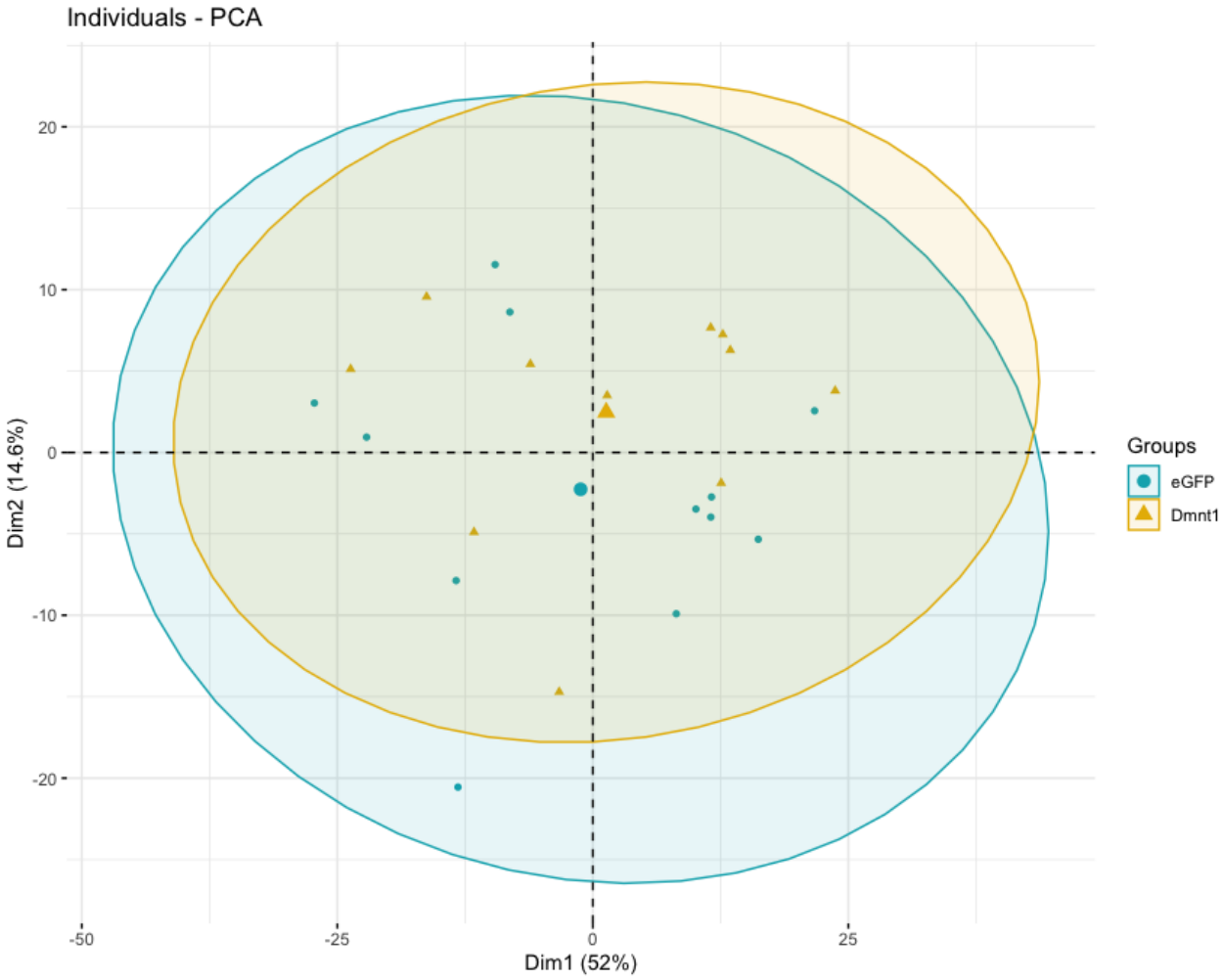


Fig 1. There was little differentiation of samples reflecting how few genes were differentially expressed between treatments. Smaller points represent individual samples and are colored by their treatment category. Large points represent the centroid (i.e., mean) of each treatment. Circles represent 95% confidence intervals.

GO term overrepresentation

The differentially expressed genes between *Dnmt1* knockdown and controls produce 87 overrepresented GO terms. The top three Biological Process terms are female meiosis I – GO:0007144, sperm axoneme assembly – GO:0007288, and negative regulation of imaginal disc growth – GO:0045571 (P = 0.00021.

P= 0.00044, P = 0.00075, respectively). Although meiosis is a top GO term, the overall list does not represent overrepresentation of meiosis terms. The list contains one meiosis specific term, five mitosis specific terms, and five terms specific to general cell development. The top three Molecular Function terms with more than one annotated gene are mRNA 3'-UTR binding - GO:000373, ribonucleoside-diphosphate reductase activity - GO:000474, and coenzyme A transmembrane transporter activity - GO:0015228 (all P = 0.0101). The top three Cellular Compartments terms with more than one annotated gene are MKS complex - GO:0036038, mitotic spindle pole - GO:0097431, and mitochondrial respiratory chain complex IV - GO:0005751 (P = 0.0108, P = 0.0162, P = 0.0162, respectively). The remainder of the terms can be found in Supplementary File 3.

Characterization of cytosine methylation

We assessed genomic distribution and abundance of cytosine methylation to try to understand its potential function for *B. tabaci*. Cytosine methylation is found almost exclusively within a CpG context, which is consistent with other Hempiteran and insects in general. Cytosines are methylated symmetrically (i.e., both strands of a CpG are methylated or not; Fig. 2), which is an indication that *Dnmt1* functions as expected. GC content of genes is higher than the background intragenic genome. This is expected because *B. tabaci* is an AT biased genome overall (genome: 39% GC, genes: 44% GC; Fig. 3A). Methylated cytosines within genic regions mirror the GC content of genes (Fig. 3B). Genes have a very bimodal distribution of methylation status (i.e., genes are either highly methylated or not; Fig. 3C). Transposons did not have consistent or high levels of methylation (Fig. 3D). Methylated cytosines are enriched within genic and exonic regions but are seen at genomic levels within introns (Fig. 4).

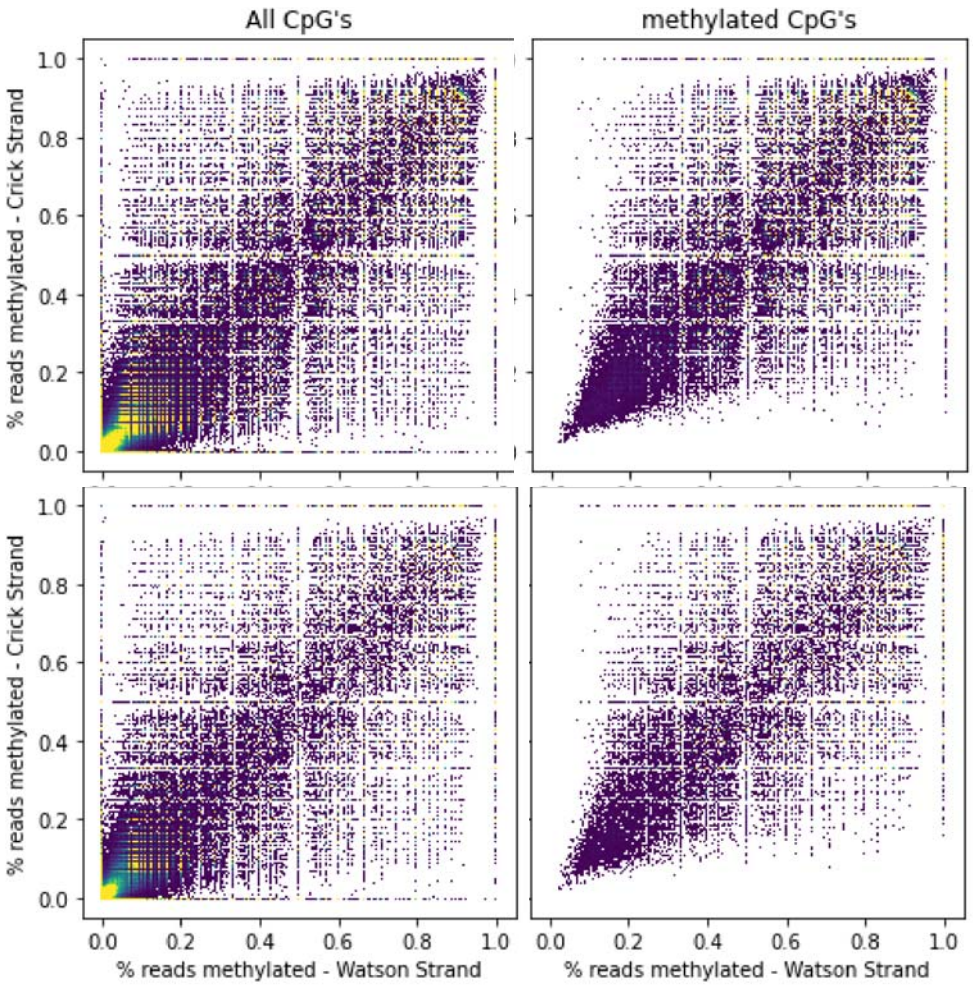


Fig 2. Cytosine methylation of *Bemisia tabaci* is highly symmetric, consistent with many other insects. Density plot of CpG's within different contexts. Lighter colors represent denser value regions.

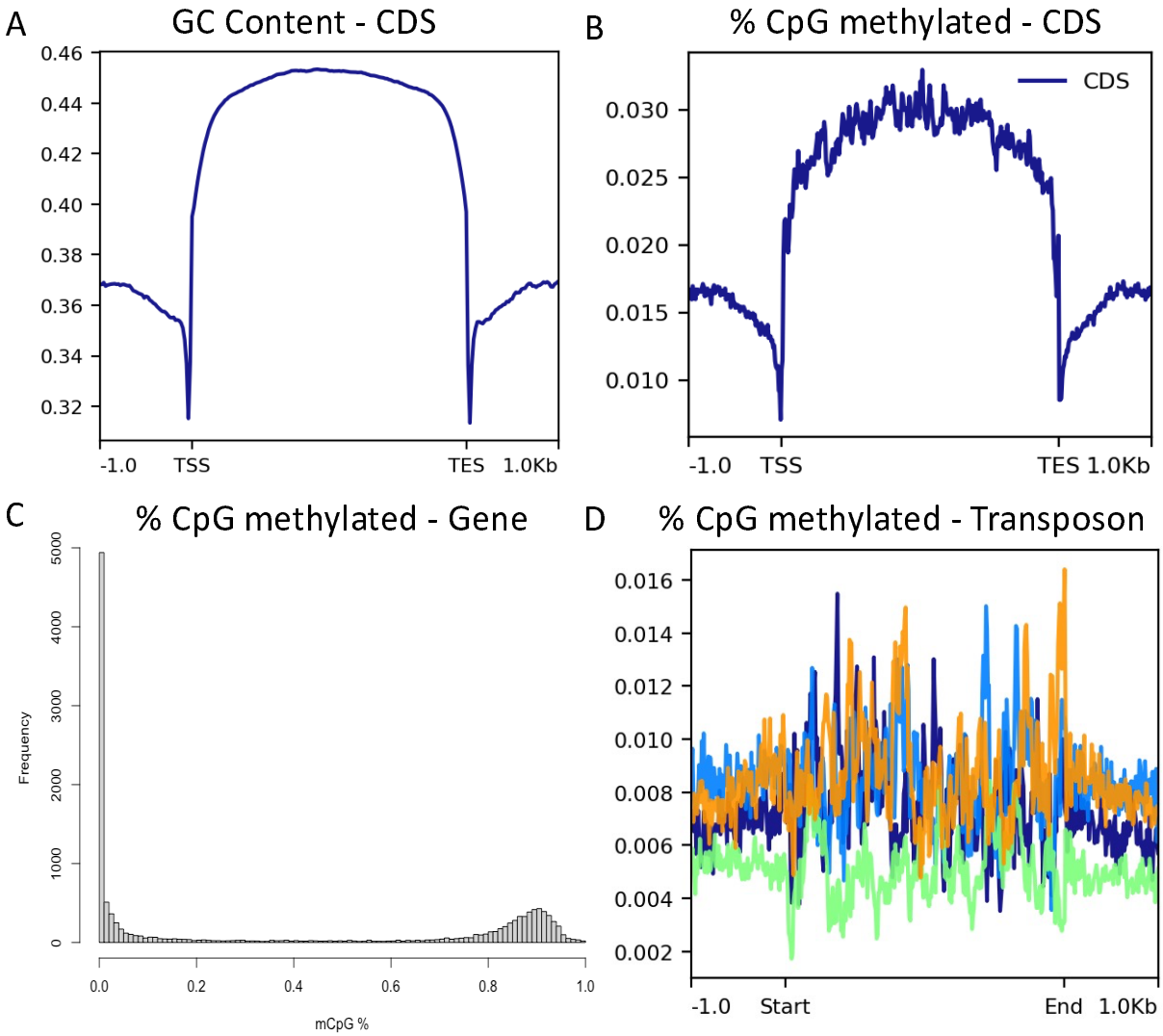


Fig 3. Cytosine methylation of *Bemisia tabaci* is mostly consistent with many observed trends of other insects. (A) The GC content increases over gene bodies. (B) CpG methylation is mostly found in gene bodies. (C) Genes are either highly methylated or non-methylated. This is a more bimodal distribution than is seen for other insects. (D) There is little cytosine methylation over the most frequent transposon within the *B. tabaci* genome.

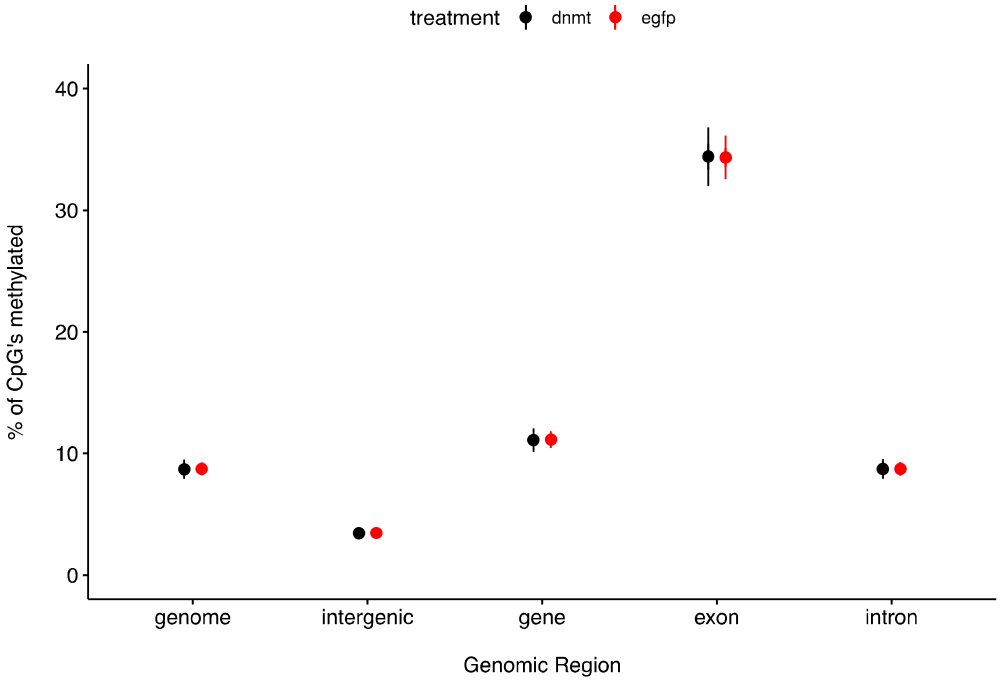
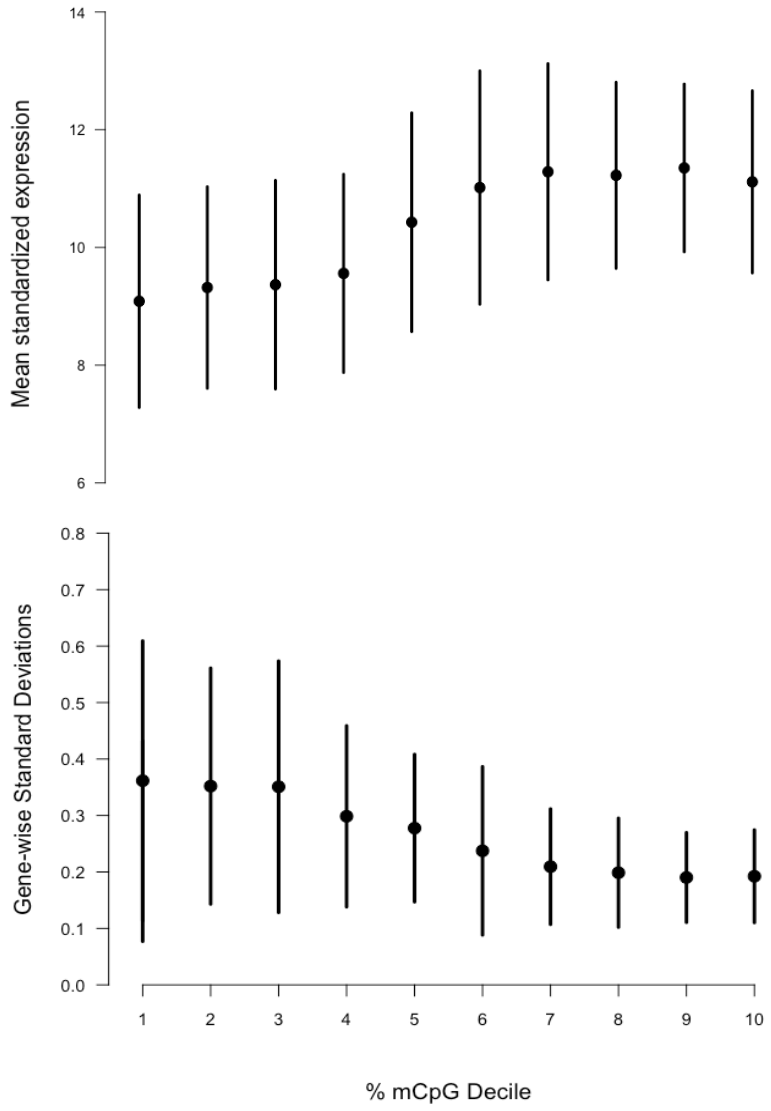


Fig 4. Cytosine methylation of *Bemisia tabaci* is not detectably influence by *Dnmt1* RNAi. Cytosine methylation is enriched with genic and exonic regions but is not detectably influenced by *Dnmt1* RNAi. Each point represents the mean ± SEM of each treatment within the labeled category.

Cytosine methylation is associated with overall gene expression (between loci differences)

High levels of gene methylation are associated with high levels and low variances of gene expression (Fig. 5). This is a well-established pattern that suggests if cytosine methylation is performing a function, it is likely similar between *B. tabaci* and other insects.



After assessing the general trends of cytosine methylation within *B. tabaci*, we assessed the effect of *Dnmt1* knockdown on gene methylation level between treatment and controls. Very few gene loci differed at all between the treatments; only 2,391 differed by 1% or more (Fig. 6). No gene was statistically significantly different after FDR correction.

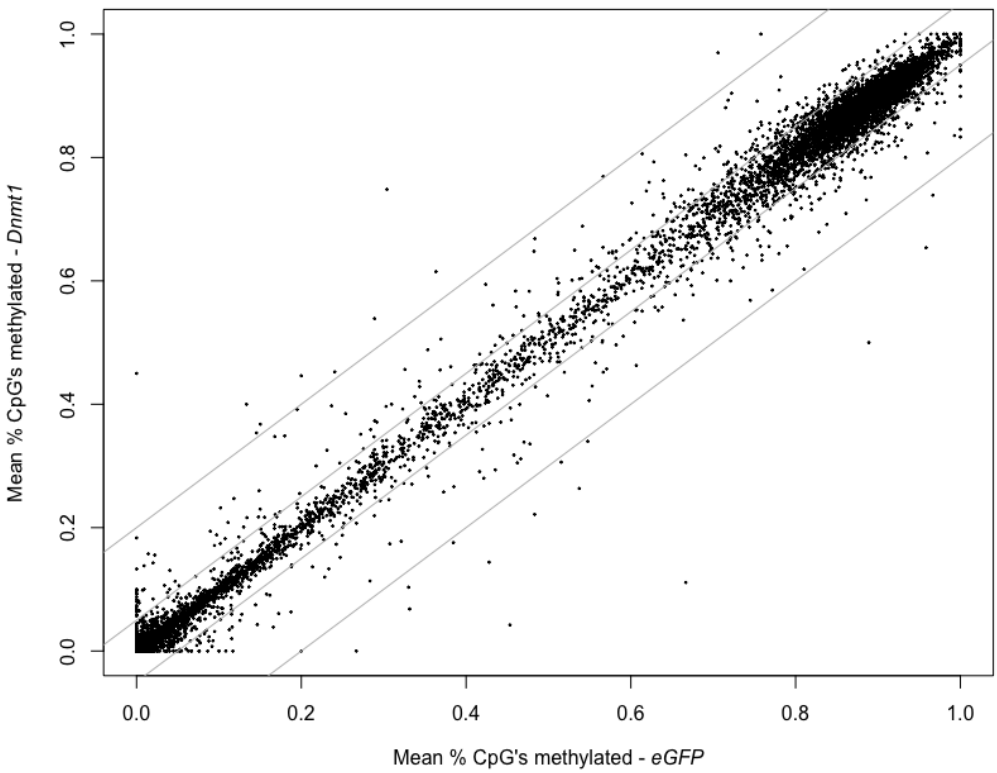


Fig 6. Very few genes differ for methylation status between treatments. The inner and outer diagonal lines represent 5% and 20% differentiation of *Dnmt1*vs *eGFP* treatments, respectively. Each circle represents an individual gene.

Differential gene methylation is not associated with differential gene expression (within locus differences)

Lastly, we assessed if the difference of gene methylation that were present influenced gene expression within (and not between as above) a gene. There was no indication that difference of methylation influenced difference of gene expression (Fig. 7). This is consistent with almost all other insects.

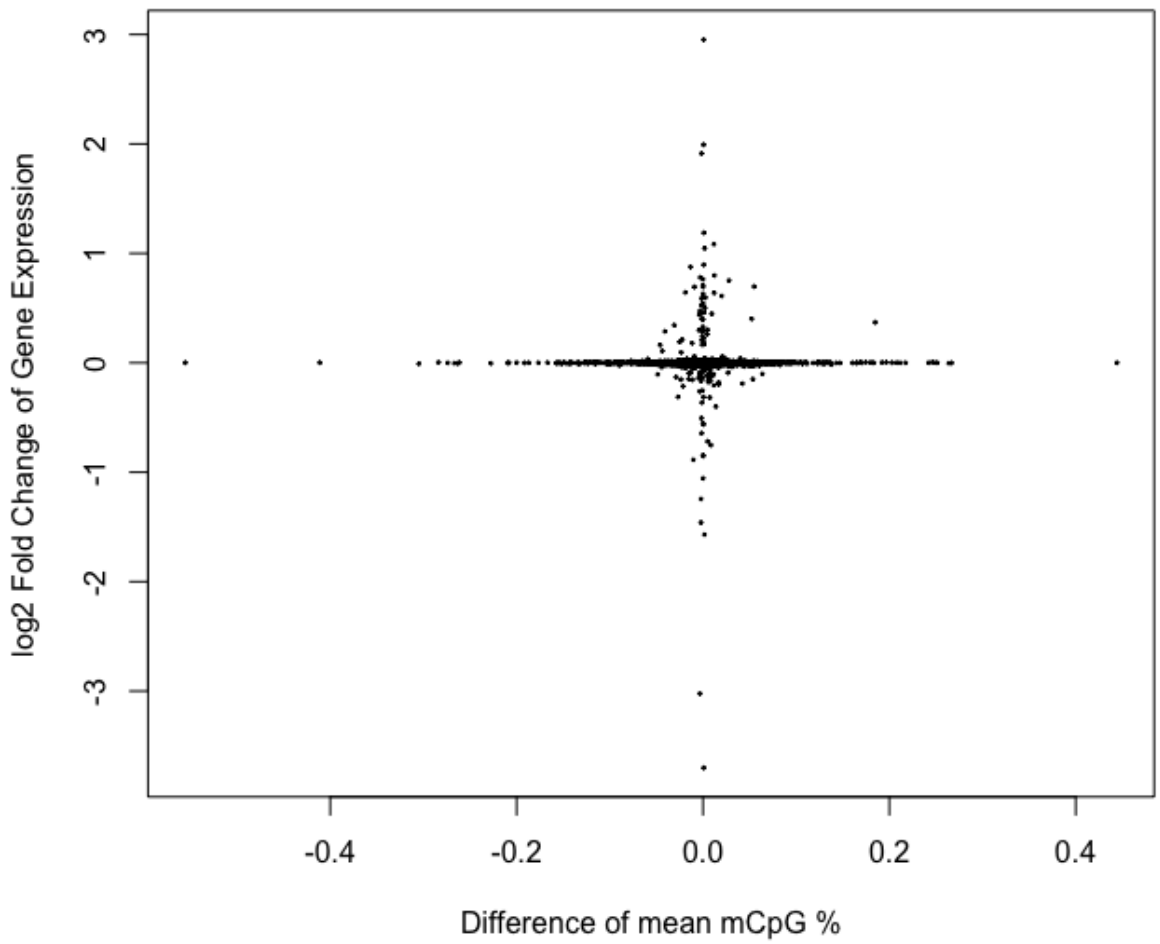


Fig 7. Differential methylation is not associated with differential gene expression. Scatterplot of differential expression against differential expression that would approximate a 1:1 relationship. Each circle represents an individual gene.

Discussion

Dmnt1 shows a baffling diversity across species of insects (Bewick et al. 2017), but one common function when *Dnmt1* is present is in gametogenesis (Zwier et al., 2012; Schulz et al., 2018; Bewick et al., 2019; Li et al. 2019; Amukamara et al., 2020; Gener et al., 2020; Ventós-Alfonso et al., 2020; Takada et al., 2021; Washington et al., 2021; Arsala et al., 2022; Xu et al. 2022; Cunningham et al., 2023; Ivasky et al., 2023; Shelby et al. 2023). Here, we investigated the genetic underpinning of severe reproductive deficits after *Dnmt1* expression was reduced using RNAi in the whitefly *B. tabaci*. This treatment causes females to produce both fewer and less viable eggs (Shelby et al., 2023). We used RNA-seq of whole-body samples to specifically test an *a priori* hypothesis that this treatment would impact meiotic molecular pathways preferentially (Amukamara et al., 202; Washington et al., 2021; Cunningham et al., 2023). We also explored this dataset to examine if other molecular pathways associated with germ cell maturation were impacted. Relatively few genes overall were differentially expressed. While our hypothesis was not strongly supported, we did find differential expression of *Wnt*-like genes. From this, we think that *Wnt* and its pathways becomes an interesting candidate pathway for future study. We also performed a

baseline characterization of genomic cytosine methylation of *B. tabaci*, the context in which it is found, and the impact of *Dnmt1* knockdown on it. Cytosine methylation was found at comparable level to other Hemipteran, within a CpG context, and was enriched within genic regions across the genome. Absolute cytosine methylation differences between loci were positively associated with gene expression level, but relative differences of cytosine methylation level within loci due to treatment was not associated with gene expression differences. While this work could have detected strong effects, we believe the lack of pervasive differential gene expression given the severe reproductive deficit phenotype is likely due to using whole bodies. This is likely attributable to overall few cellular divisions happen at the whole-body level, but are needed for *Dnmt1'*s reduction to manifest its effect including mitotic gametes and some other high-turnover tissues, e.g., certain digestive tissues. The ability to detect differential gene methylation were also likely affected by the few cells within our sample population. Moving forward, there is a need to take a tissue specific approach to understanding the genetics of this phenotype. Our analysis also provides a new candidate pathway for the study of reproductive deficits of female insects, increased the understanding of the genetics of insect reproduction, and provide a resource for studies of genomic methylation of *B. tabaci*.

As expected, given previous research (Bewick et al., 2019, Cunningham et al., 2023), despite the dramatic phenotype, we did not find large numbers of differentially expressed genes. This suggests that *Dnmt1* knockdown is affecting specific genes or gene networks, rather than producing the reproductive deficit by instigating a global transcriptional response. Our *a priori* hypothesis that meiotic-specific pathways would be the more affected compared with mitosis or general cell cycling pathways did not receive strong support overall. None of our meiosis candidate genes was differentially expressed compared with one or two in the other categories – general cell proliferation, cell cycle, and mitosis. Genes that were annotated with the GO term of meiosis were not enriched in the list of all genes ranked by expression differences that also had assigned GO terms, regardless of whether they differentially expressed or not. Among the differentially expressed genes themselves (96 genes), one meiosis GO term was overrepresented. However, the list also contained several mitoses specific and development specific GO terms. We believe this general overrepresentation of development genes is an overall reflection of assaying a perturbed reproductive tissue and not specific of any pathway being preferentially affected. Thus, we did not find support for *Dnmt1* being directly involved in the control of meiosis.

However, we found intriguing evidence that a conserved genetic pathway, the *Wnt* pathway, might be affected by *Dnmt1* knockdown. *Wnt*'s of insects are usually viewed as general development genes; involved with cell proliferation, migration, patterning, and idenity (Murat et al., 2010). In human colon cancer cells, DNMT1 is known to influence the WNT pathway via negative regulation of its members *β-catenin* and *Snail 1* through a DNA methylation-independent mechanism (Espada et al., 2011). While mammalian DNA methylation operates differently than it does for insects, these results generate specific, testable hypotheses about how the expression of *Dnmt1* is required for proper regulation of gametogenesis. The regulation of the *Wnt* pathway by *Dnmt1* being methylation independent also aligns with much of the evidence produced by insect systems (Bewick et al., 2019; Amukamara et al., 2020; Ventós-Alfonso et al., 2020; Washington et al., 2021; Arsala et al., 2022; Cunningham et al., 2023; Ivasky et al., 2023; Shelby et al., 2023). This pathway has also been shown to be involved in entry into meiosis of mice (Chassot et al., 2011, Le Rolle et al., 2021). Future experiments will explore the interaction between the *Wnt//β-catenin* and *Dnmt1* in our system.

One possible explanation for detecting few differential expressed genes is that we were not sampling only the tissue in which *Dnmt1* knockdown has its effect, the ovaries, but rather sampling whole

bodies. However, *Dnmt1* expression was measurably reduced, and this treatment is known to reduce the presence of its protein, DNMT1, within the nucleus of ovarian cells of *Dnmt1* knockdowns (Shelby et al., 2023) and this magnitude of differences is reasonable from whole bodies of this species when the experimental effects are regional or tissue specific (Tadmor et al., 2022). A second possible explanation is that we did not detect high levels of differentially expressed genes because we did not appreciably reduce DNA cytosine methylation. The reduction in DNA methylation with loss of *Dnmt1* requires cell division and DNA synthesis. High turnover tissues exist within adult insects, such as digestive and reproductive tissues. However, they would be the minority of the cell pollution sampled here. Thus, our bisulfite sequencing data might not detect differential DNA methylation within the small proportion of cells that divided between treatment and sampling. However, given the consistent result that changes of cytosine methylation pattern are not associated with differential expression in *O. fasciatus* gonads (Bewick et al., 2019, Cunningham et al., 2023), our data support the hypothesis that *Dnmt1* knockdown is affecting targeted genetic pathways in the gonads to specifically affect gametogenesis. This adds to the evidence that *Dnmt1* or lack thereof is acting through a cytosine methylation independent pathway. The gametes/eggs that were affected were post-meiotic and such cells would have similar cytosine methylation patterns to control samples; however, they lacked nuclear DNMT1. Recently, an ancestrally duplicated domain of DNMT3 of honey bees showed binding to histone residues, a previously uncharacterized interaction within insects (Kucharski et al., 2023). Reanalysis of the alternative splicing of this gene during development also showed many isoforms produced with the duplicated domain and without the catalytic methyltransferase domain (Kucharski et al., 2023). This supports the general idea that these *Dmnt* gene families are more diverse than previously appreciated and that they have the capacity to act via different pathways. This would suggest that *Dnmt1* knockdown effects are not likely being mediated through difference of cytosine methylation.

Another major aim of the study was to characterize the cytosine methylation of *B. tabaci*. This was both a general characterization and what influence the RNAi treatment might have on the patterns. Cytosine methylation is found within a CpG context and enriched within genic regions. Genes have a bimodal distribution whereby gene are either highly methylated or not and methylation level is associated with gene expression level between loci. All of these patterns are common among insects and suggest that cytosine methylation is comparable among hempiterans (Bewick et al., 2017; Provataris et al., 2018). There was little cytosine methylation found across transposons, which suggests that this nucleotide modification is not serving as a defense against those elements (Bewick et al., 2019; Gladstad et al., 2019). The RNAi *Dnmt1* treatment produced no differentially methylated genes. We believe the same explanation for few differentially expressed genes applies to this result as well – whole body samples were not sensitive enough to detect a change of gene methylation within a minority of cells within our samples. Although methylation level between treatments were not statistically significantly different, some loci did show differences. However, there was no indication that these differences collectively were associated with gene expression levels. This also makes *B. tabaci* comparable to other insects where an association between difference of gene methylation and expression is rare (Moradin and Brendel, 2021; Dixon and Matz, 2022; Duncan et al., 2022; Malezka and Kucharski, 2022). Overall, *B. tabaci* shows many conserved patterns of cytosine methylation of insects but a more tissue specific approach is warranted before making definitive conclusions.

Pest management of *B. tabaci* is one of the primary reasons for any work with this species. Our results here are add to the understanding of its reproduction and biology and will inform experimental design moving forward. Clearly, *Dnmt1* knockdown produces severe reproductive deficit for female *B.*

tabaci (Shelby et al., 2023). We also now know that *B. tabaci* has cytosine methylation patterns and association like other hemipteran and insects meaning we can use those species to help inform hypotheses, predictions, and conclusions. Our work also suggests that we need to focus on disrupted tissues themselves with standard whole body experimental designs of the field failing here. However, any work that informs the understanding of genetics that underpins *B. tabaci* reproduction and reproductive disruptions is worth pursuing.

Experimental Procedures

To understand the functional role that *Dnmt1* plays during gamete formation of female *B. tabaci*, we used RNAi to knockdown *Dnmt1* gene expression replicating the protocol of Shelby and colleagues (2023). After the treatment, we tested two sets of *a priori* predictions after gene expression quantification using RNA-seq; one using candidate genes and the other using Gene Ontology terms. The same RNA-seq data were also used to perform exploratory analyses to assess how *Dnmt1* knockdown perturbed the transcriptome of females. We also assessed cytosine methylation of both control and RNAi-treated females. We first characterized the level *B. tabaci* has genomic methylation, in what genomic context it is found, how gene methylation related to gene expression globally. We then assessed if *Dnmt1* expression reduction caused differential gene methylation and if differential gene methylation was associated with differential gene expression.

Animal colony and husbandry

This experiment used the same laboratory population and husbandry protocols as Shelby and colleagues (2023). Briefly, these *B. tabaci* colonies were started from population collected from a cotton (*Gossypium hirsutum*) field site in Tift County, Georgia, USA in 2018 (McKenzie et al., 2020) belonging to the MEAM1 *B. tabaci*. The whiteflies were maintained on collard (*Brassica oleracea*) plants using individual pots and grown to a minimum height of 15 cm. Both whiteflies and collards were maintained at 26°C with a 14:10 hour light:dark photoperiod in 51 cm × 28 cm × 28 cm aluminum insect cages (Bioquip Products, Rancho Dominguez, CA, USA) within a Percival Incubator (Percival Scientific, Perry, Iowa, USA) during the entire experiment.

RNA interference (RNAi) synthesis, administration, and quality control

We created RNAi constructs of *Dnmt1* following Shelby and colleagues (20123). Briefly, RNAi constructs of *Dnmt1* were prepared via PCR using 500 ng of RNA from unmated females. The construct was targeted towards the Replication Fork Domain (RFD) motif the *Dnmt1* gene. Afterwards, double stranded RNA was synthesized with an Ambion MEGAscript kit (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. This reaction was purified with a phenol:chloroform:IAA extraction followed by a sodium acetate precipitation. We measured concentration using Invitrogen's Qubit system with the ssRNA kit following the manufacturer's protocol. We used *eGFP* as an exogenous control construct.

Females were haphazardly allocated to treatment group. Newly-eclosed females were removed from colonies within 24 hours post-eclosion to drastically reduce the opportunity to mate. Adult female *B. tabaci* at 3-6 days post-ecolsion were fed RNAi solution of either *Dnmt1* or *eGFP* each at a concentration

of 0.05 µg/mL within a 10% w/v sucrose solution contained within a cap of a 1.7 mL microcentrifuge tube. The solution was colored with 200 µl of green food coloring. Parafilm was placed over the cap to provide a membrane for feeding. From 75–100 female whiteflies were placed into each prepared acetate tube, which was then wrapped with black construction paper to promote movement to the top of the tube. Females were allowed to feed for 24 hours, and consumption was confirmed by observing a green abdomen before collection. Control and *Dnmt1* females were flash frozen with liquid nitrogen and stored at −□80°C until nucleotide extraction. Twenty-five females were pooled into each biological replicate.

This treatment does not impact lifespan between control and *Dnmt1* treated females or gross morphological structures (Shleby et al., 2023). This treatment statistically significantly reduces the expression of *Dnmt1* of treated females (Shelby et al., 2023).

RNA & DNA extraction

Total RNA and genomic DNA was extracted using a Qiagen Allprep DNA/RNA Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's protocol. Homogenization of the females was performed using a Bead Bug (Benchmark Scientific, Sayreville, New Jersy, USA) at 4000 rpm for 60 seconds. Quantification of DNA and RNA was done with a Qubit fluorometer using the DNA HS and RNA BR kits, respectively, following the manufacturers protocol.

RNA-seq High-throughput library preparation and sequencing

A sample of 150 ng of extracted total RNA of *B. tabaci* was used to construct poly-A selected, non-stranded Illumina compatible libraries by Novogene Corporation (Sacramento, CA, USA). We targeted 20M 2 x 150 bp read pairs per biological replicate using an Illumina NovaSeq by Novogene.

RNA-seq quality control and mapping

Reads were initially assessed for quality with fastQC (v0.11.9; default settings; Andrews, 2010). Reads had adapters trimmed with cutadapt (v2.8; --trim-n -O 3 -u -2 -U -2 -q 10,10 -m 30; Martin, 2011) using the TruSeq adapter sequences. Afterward, reads were reassessed with fastQC with default settings. Overlapping reads were merged with FLASh (v 1.2.11; default settings; Magoc and Salzberg, 2011). As a final QC step, reads were mapped to rRNA genes and were removed with SortMeRNA (v4.3.3; s rRNA: NC_006279, l rRNA: NC_006279.1; Kopylova et al. 2012). We used the NCBI RefSeq assembly (ASM185493v1; Chen et al., 2016) and annotation (GCF_001854935.1). HISAT2 (v2.2.1; no soft-clipping; Kim et al. 2019) was used to map reads to the genome. Mappings were converted to read counts by StringTie (v2.1.7; Pertea et al. 2015) following the manual instructions for export to DESeq2.

Functional annotation

We added functional annotation to the *B. tabaci* proteome/transcriptome using eggNOG-mapper webserver using the Auto taxonomic scope setting (http://eggnog-mapper.embl.de/; v 2.19; Cantalapiedra et al. 2021). This annotated 16,813 of 22,737 protein/transcript models (74.1%) with Gene Ontology terms, which was the database to perform our GO term enrichment analysis and GO term overrepresentation analysis (Supplementary File 1).

a priori candidate gene screen

We tested our hypothesis that *Dnmt1* knockdown would preferentially influence meiotic, not mitotic, or general cell cycle, pathways by assessing a series of candidate genes associated with each pathway. These genes were chosen as canonical members based on literature searches (Cunningham et al., 2023). These include cell fate and proliferation genes that we did not expect to be differentially expressed (members of the *Wnt*, *Frizzled*, and *Pumillio* families), cell cycle control genes (some of which we did expect to be differentially expressed (*Vasa*) and some of which we did not expect to be differentially expressed (*Cyclin B3*, *Cyclin D2*, *Cyclin D3*, *Cell Division Cycle 20*, *Cell Division Cycle 25* family members)), maintenance of chromosome genes that we did expect to be differentially expressed (*Structural Maintenance of Chromosome 3* family members), meiotic transition gene that we did expect to be differentially expressed (*Boule*, *Decapentaplegic*, and *Glass boat bottom* familes), and meiotic recombination genes that we expected to be differentially expressed (*SPO11 Initiator of Meiotic Double Stranded Breaks*, *MutS Homolog 5*, *Meiotic Nuclear Divisions 1*, and *Homologous-Pairing Protein 2* families). We did not predict specific directional changes, except decreased expression of *Vasa* and *Boule* within the *Dnmt1* knockdowns. We extracted raw P values for differential gene expression from DESeq2 and compared them with Adaptive Two-Step B-H Thresholding test, which is less conservative than standard Benjamini-Hockberg correction (Benjamini et al., 2006).

a priori Gene Ontolog (GO) term enrichment analysis

We also tested our hypothesis using a very broad test of pathway enrichment. We selected five high-level GO terms representing the molecular pathways that we predicted would or would not be after *Dnmt1* knockdown. These were GO:0007049 Cell Cycle, GO:0000278 Mitotic Cell Cycle, GO:0051321 Meiotic Cell Cycle, GO:0007276 Gametogenesis, and GO:0007283 Spermatogenesis. With our previous observed phenotypes, we expect Meiotic Cell Cycle, Gametogenesis, and Spermatogenesis to be enriched. We expected Cell Cycle and Mitotic Cell Cycle to show no enrichment. We included GO:0070265 Necrotic Cell Death and GO:0006915 Apoptotic Process to assess if our samples were undergoing these processes. We used the S values from DESeq2 to rank order genes. We used fgsea R package (Korotkevich et al. 2021) with default parameters to test if genes annotated with these GO terms were enriched at the head of these lists. The same R package was used to visualize the results.

Differential gene expression

Read counts were imported into R using tximport (Bioconductor v1.20.0; Soneson et al. 2015) following the manual's instructions. We used R (v4.1.0; R Core Team, 2021) within an RStudio IDE (build 492; RStudio Team, 2022) for the analysis.

DESeq2 (Bioconductor v1.32.0; default settings; Love et al. 2014) was used for the differential gene expression analyze following the manuals suggestions for exploratory data analysis and sample/analysis quality control. *eGFP* was set and used as the comparison group. We checked the model matrix specification to ensure correct specification (i.e., that program was contrasting the samples in the correct way). After importing and initial analysis, samples were plotted with a PCA to visually check for outliers according to the manual's recommendation. After inspection, we removed one *Dmnt1* sample that

was both an intra-group PCA outlier and having very high expression of *Dnmt1* that suggested the RNAi had not worked properly.

After removal of the outliers the analysis was repeated using the same settings of DESeq2 and the results were again quality controlled. We used the default dispersion estimator and shrinkage method, apeglm (Zhu et al. 2019). We used s-values to estimate statistical significance after false discovery rate correction at the level of 0.05 (Stephens 2016). Results were visualized with the fviz_pca_ind function of the factoextra R package (Kassambara and Mundt 2020).

Gene Ontology (GO) term overrepresentation analysis

We found overrepresented GO terms among the differential expressed gene to provide us with biological processes that might be perturbed using the topGO package of R (v2.48.0; Alexa et al. 2006). Often termed an enrichment analysis (Huang et al. 2009), the analysis specifically tests for overrepresentation of a GO term from a cohort of provided genes (usually those that are differentially expressed) compared with the expectation of the same number of random picks from all expressed genes. We performed these GO term tests using Fisher's exact test with the weighted algorithm.

Whole-genome bisulfite sequencing (WGBS) library preparation and sequencing

A sample of 150 ng of genomic DNA was used to construct bisulfite treated genomic DNA Illumina compatible libraries by the Novogene Corporation (Sacramento, CA, USA). Lambda phage gDNA, which is known to be completely unmethylated, was added to these libraries to provide a negative control and estimate conversion efficiency. We targeted 30X coverage of the genome using 2 x 150 bp pair-end reads. Sequencing was performed on an Illumina NovaSeq.

WGBS quality control, mapping, and cytosine methylation analysis

As described above, reads were quality assessed with fastQC, trimmed with cutadapt using the TruSeq adapter sequences, and reassessed with fastQC with default settings. Reads were mapped to the NCBI RefSeq assembly (ASM185493v1) & annotation (GCF_001854935.1) using HISAT-3N (v2.2.1; C to T base change, uniquely mapped non-clonal reads; Zhang et al., 2021; Supplementary File 2). We then used HISAT-3N to generate a per nucleotide results table for all cytosines and a separate one for cytosines within a CpG context, which is were almost all of cytosine methylation is found for insects (Bewick et al., 2017). Using the HISAT-3N results tables per sample, we first filtered out cytosines that had fewer than five mapped reads. We then used Python and the numpy, scipy.stats, statsmodel.stats, and pandas libraries to generate per nucleotide result tables of percent reads that indicated methylation, total mapped reads, and one-tailed binomial tests with FDR-corrected P values for each sample ($P < 0.016$, which was the average non-conversion rate of reads mapped to the lambda phage controls). Methylation status of each cytosine was collected into a per gene results table for all samples using bedtools (Quinlan and Hall, 2010) and pandas. We also generated a results table that contained only CpGs that were symmetrically mapped above our threshold. To be able to characterize cytosine methylation over repeats and transposons, we identified transposable elements using RepeatMasker (v4.1.2; Smit et al., 2013) which was consistent with the invertebrate repeat library from Repbase (Jurka, 1998) following Bewick and colleagues (2019). Cytosine methylation over these genomic regions was collated with bedtools.

Characterization of cytosine methylation

We characterized the genomic context of cytosine methylation of the *B. tabaci* in several ways. We first characterized the GC content of the genome using bedtools. We then calculated the percent of cytosines that were methylated for several genomic regions: the genome as a whole, intragenic regions, genes, exons, and introns. Lastly, we characterized percent of cytosines methylated across genes and transposons with bedtools and plotted with deepTools2 (v3.5.1; Ramirez et al., 2016).

Differential gene methylation and analysis

After understanding the genomic context of cytosine methylation, we focused on understanding its association with gene expression. First, we expected to see an association between gene methylation and gene expression such that highly methylated genes were also the highest expressed genes (Duncan et al., 2022). To that end, we divided genes into deciles and plotted those values against the mean standardized expression of the gene within each decile using R. We also plotted how differences of gene methylation were associated with gene expression standard deviations.

To understand the effect of *Dnmt1* RNAi on gene methylation, we tested for statistically significantly differences of gene methylation levels between the two treatments using the t.test function of R. Before this test, we filtered the results for genes that showed at least a 1% difference for percent of cytosines methylated (2,391/13,901 genes) so the *t* test would not return an error due to data showing no variation. We plotted the mean percent cytosines methylated for each gene between the two treatments.

Lastly, we assessed how differences of gene methylation were associated with difference of gene expression by plotting log2 Fold Changes of gene expression by difference of mean percent methylated cytosines.

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Author Contribution Statement

CBC, PJM, AJM, EAS, and AS designed the study. EAS proposed examining the *Wnt* genetic pathway. EAS and ECM performed the experiments. CBC analyzed the data. CBC, PJM, AJM, and EAS interpreted the data. All authors contributed to drafting the manuscript.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Data Availability

All high-throughput data is available under NCBI BioProject # XXXX

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