DNMT1 IS MORE THAN A METHYLTRANSFERASE. JUST ASK WHITEFLIES.

by

EMILY ANNE SHELBY

(Under the Direction of Patricia J. Moore)

ABSTRACT

Genome stability refers to the ability of an organism's DNA to maintain its structure and function over time. DNA methylation is an epigenetic mechanism that is important for genome stability despite its primary function being unknown. DNA methyltransferases, specifically DNA methyltransferase 1 (DNMT1), is vital for reproduction and development across living organisms. *Bemisia tabaci* (Order: Hemiptera), also known as the silverleaf whitefly, has become a top global pest owing to its incredible reproductive potential and genetic machinery. Their recalcitrant nature has necessitated exploration of control methods other than insecticides. The following work explores the relationship between DNMT1 and insect reproduction in hopes to inform future whitefly management strategies and begin to disentangle the complex relationship between DNMT1, DNA methylation, reproduction, and genome stability. The first half of this dissertation reviews the current state of knowledge for both *Dnmt1* and whiteflies and lays the foundation for *Dnmt1* as an ideal candidate gene for targeting whitefly management based on specificity and sustainability considerations. The latter half investigates the role of *Dnmt1* in whitefly oocyte and embryo development. We found that the reduction of DNMT1 resulted in reduced egg production and loss of egg viability without reduced survival or a significant

reduction in methylation levels. This suggests that DNMT1 plays a specific role in normal oocyte production and oocyte health outside of its canonical role as a methyltransferase. When we investigated the phenotypic effects of *Dnmt1* knockdown on embryogenesis, we found that loss of DNMT1 disrupts blastoderm formation and nuclei morphology, suggesting that DNMT1 likely plays a role in cell cycle regulation during early embryogenesis. These results conclude that targeting whitefly DNMT1 results in significant population reduction through loss of reproductive capabilities while relaxing selective pressures, making it a viable option for further development as a pest management product. The results of this dissertation also shed light on DNMT1's role as a key player in genome stability.

INDEX WORDS: *DNA methyltransferase 1*, *Bemisia tabaci*, whitefly, insect, RNA interference, egg, embryo, reproduction, development

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DEDICATION

This dissertation is dedicated to my leasing manger, Gracie Truluck, who unknowingly summed up the story of my life in an aggressive email about raising my rent:

> You are lucky to have made it this far at that rate.

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CHAPTER 1

INTRODUCTION

1.1 DNA methylation as a mechanism for genome stability

Genome stability refers to the ability of an organism's DNA to maintain its structure and function over time. Because preservation of the genome is critical for cell function and accurate transmission of genetic material from one generation to the next, numerous mechanisms, such as DNA repair responses, cell cycle checkpoints, chromatin structure, and epigenetic modifications all contribute to genome stability by working in tandem to ensure that DNA is intact, functioning, and complete (Brown & Robertson, 2007; Cuozzo et al., 2007). One of the most studied mechanisms for genome stability is the epigenetic modification DNA methylation. The most common type of DNA methylation involves the addition of a methyl group (CH3) from S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue of a DNA molecule, producing 5-methylcytosince (Moore, Le, & Fan, 2013). The methylation reaction is catalyzed by enzymes called DNA methyltransferases (DNMTs; Goll & Bestor, 2005). Following the addition of the methyl group, the geometry and physico-chemical properties of DNA are altered (Li, Peng, & Panchenko, 2022). These changes modulate DNA accessibility, which affects how other proteins, such as those involved in dna repair, replication, and transcription, interact with DNA (Li, Peng, & Panchenko, 2022). Due to its importance in genome stability, DNA methylation in humans has been suggested to play vital roles in processes such as aging (Unnikrishnan et al., 2019;

He et al., 2021) , fetal development (Ghazi et al., 2021; Bestry et al., 2022; Saini et al., 2022), and cancer biology (Chen et al. 2022).

1.2 The primary function of DNA methylation

Although DNA methylation studies have been at the forefront of epigenetics for over thirty years, it's primary role (that is to say, the role that led to its widespread distribution across the tree of life) has been hotly debated (Mattei, Bailly, & Meissner, 2022). The first proposed roles for DNA methylation were made based on findings from bacterial studies 23 years after the discovery of 5-methylcytosine in bacteria (Johnson & Coghill, 1925; Vischer & Chargaff, 1947; Hotchkiss, 1948). It was shown that 5-methylcytosine provided defense against phages as methylated bacterial DNA is protected against damage via restriction enzymes (Gold, Hurwitz, & Anders, 1963; Arber, 1965). In addition, a link between bacterial DNA methylation and DNA replication was observed, where deficiencies in methyl donors led to DNA degradation and replication defects (Billen & Hewitt, 1966; Billen, 1968; Lark, 1968). The revelation that DNA methylation is a widespread phemenon led to speculation about the role of DNA methylation in higher organisms, including that DNA methylation acted as a transcriptional regulator (Comings, 1972). Indeed, the reigning hypothesis became that DNA methylation regulated gene expression and therefore played a vital role in development (Holliday & Pugh, 1975; Riggs, 1975; Sager & Kitchin, 1975). This hypothesis was strengthened by the understanding of transcription-related phenomena such as genomic imprinting and X chromosome inactivation in mammals (Lee & Bartolomei, 2013; Ferguson-Smith & Bourchis, 2018; Galupa & Heard, 2018). Then, in 1995, it was suggested that the primary role of DNA

methylation is transcriptional repression, and that DNA methylation was the causative agent for the evolutionary increase in the number of genes observed in vertebrates (Bird, 1995). Indeed, the appearance of global methylation levels in organisms corresponds with the origin of vertebrates (Tweedie et al., 1997). However, the correlation between DNA methylation and gene number became less evident as more sequenced genomes became available (Field et al., 2004). In addition, loss of DNA methylation machinery had been shown to lead to misexpression of only a very limited number of genes (Field et al., 2004). An alternative view arose that the primary function of DNA methylation is to suppress transposable elements, with gene expression regulation being either second, or illusory (Yoder et al., 1997; Walsh & Bestor, 1999). This hypothesis was strongly argued against, with detractors citing the absence of transposon silencing in germline cells as evidence against its credibility (Bird, 1997).

There is still no unifying theory for the function of DNA methylation. Although many studies have shed light on how methylation impacts transcription and interfaces with the histone code in mammals, far less is known about how it regulates genome stability in those systems. It should also be noted that most of the hypotheses regarding DNA methylation's primary role has been gleaned from results in the highly-derived mammalian systems. Despite this, the old DNA-methylation-only-controls-gene-expression dogma persists.

1.3 The DNA methylation system in insects

The patterns of DNA methylation and its related machinery is fundamentally different in insects, which has led to questions about the wider evolutionary significance

of DNA methylation (Mattei, Bailly, & Meissner). DNA methylation levels are highly variable in insects (Bewick et al., 2017). In Diptera, including the model *Drosophila melanogaster*, DNA methylation is almost non-existent with barely detectable levels of DNA methylation (less than 1%; Rae & Steele, 1979; Urieli-Shoval et al., 1982). DNA methylation has also been lost in specific lineages in Coleoptera, Lepidoptera, and Hymenoptera (Bewick et al., 2017; Provataris et al., 2018; Lewis et al., 2020). In orders that do have methylated DNA, such as Hemiptera and Blattodea, the levels are variable and cannot be predicted based on phylogeny (Bewick et al., 2017; Provataris et al., 2018; Lewis et al., 2020). Studies in eusocial insects have argued for its role in caste development by regulating gene expression and splicing (Elango et al., 2009; Bonasio et al., 2012; Yan et al., 2014;Patalano et al., 2015; Yan et al., 2015). However, such findings are not always consistent across studies, (Libbrecht et al., 2016; Standage et al., 2016) and have therefore remained controversial. There are also clear differences in the patterns of DNA methylation between insects and vertebrates. For example, DNA methylation in insects is restricted to the transcribed regions of genes (Feng et al., 2010; Lyko et al., 2010; Bonasio et al., 2013; Bewick et al., 2017; Provataris et al., 2018) while DNA methylation in mammals is typically localized to 5' regulatory regions (Sarda et al., 2012). There is also variation in the presence or absence of DNMTs in insects, with *Dnmt* genes being duplicated in some lineages while being completely absent in other (Bewick et al., 2017; Provataris et al., 2018). Despite some insects lacking DNA methylation, insects in those taxa still regulate gene expression. This indicates that DNA methylation may not be necessary for gene expression in all insects. Indeed, several studies have shown that there is no association between differences in DNA methylation and gene expression in insects (Libbrecht et al.,

2016; Cunningham et al., 2019; Arsenault, Hunt, & Rehan, 2018; Morandin et al., 2019). In many cases loss of DNA methylation is not fatal (Bewick et al., 2019; Ivasky et al., 2023) suggesting that DNA methylation may not be vital for survival The question then becomes “If not gene expression, then what?”

1.4 DNA methyltransferase 1: A path forward

Arguably, the best way to disentangle the complex evolutionary relationship between DNA methylation, genome stability, and gene expression is to study DNA methyltransferase 1 (DNMT1). DNMT1 is described in the literature as the maintenance methyltransferase based on initial studies reporting higher activity with hemimethylated sites compared to unmethylated sites (Pedrali-Noy & Weissbach, 1986). Like DNA methylation, DNMT1 is evolutionarily conserved and is present in virtually every major biological group (Schmitz et al., 2017). Almost universally, DNMT1 plays a role in reproduction. DNMT1 is required for embryonic development in mammals (Li et al., 1992; Lei et al., 1996; Takebayashi et al., 2007), amphibians (Stancheva et al., 2001), fish (Martin et al., 1999; Rai et al., 2006) and insects (Zwier et al., 2012;Schulz et al., 2018; Bewick et al., 2019; Amukamara et al., 2020; Ventós-Alfonso et al., 2020; Washington et al., 2021; Arsala et al., 2022). Specifically, loss of DNMT1 results in embryo death before gastrulation (Takebayashi et al., 2007; Zhang et al., 2015; Kent et al., 2016; Ventós-Alfonso et al., 2020; Arsala et al., 2022). Despite its name DNMT1 also appears to function in pathways outside of mere DNA methylation, such as DNA repair, cell cycle control, apoptosis, and RNA binding (Svedružić, 2011). Though it has been speculated in mammals that the majority of DNMT1’s function lies outside of DNA methylation (Brown &

Robertson, 2007; .Li et al., 2015; Espada et al., 2012; Mohan et al., 2022), it is difficult to distinguish methylation-independent from methylation-dependent functions in mammalian systems because both loss of DNA methylation and loss DNMT1 function results in detrimental effects. In insects, however, the decoupling of DNA methylation and DNMT1 allows for DNMT1 functions to be observed outside of DNA methylation. The strongest support for this is the case of the DNA methylation-lacking beetle *Tribolium* where loss of DNMT1 did not affect survival but resulted in the inability to reproduce (Schulz et al. 2018). Thus, the study of DNMT1 function in insects stands to be the most fruitful avenue towards an understanding of DNMT1's primary role and the evolutionary significance of the DNA methylation system.

Within this dissertation, I describe my work investigating the role of DNMT1 in insect reproduction and development using *Bemisia tabaci*, the sweetpotato whitefly. Using RNAi, gene expression analyses, various life history and viability assays, sex ratio assays, Fast^{M}C, and confocal microscopy, I establish the functions of DNMT1 in whiteflies. In addition to creating a framework for the use of *Dnmt1* as a target gene for whitefly pest management, my findings substantiate the claim that ensuring genome stability, not regulating gene expression through DNA methylation, is the main role of DNMT1 in insects.

Chapter 2 is a review that lays the foundation for the applied implications of my research as well as sets up my rationale for the use of *B. tabaci* as a study organism. First, I describe the key life history attributes of *B. tabaci* that make it a powerful pest. Then, I review the current state of RNAi-mediated pest management strategies and considerations

for successful strategies. I end the review by suggesting genes, including *Dnmt1*, that would be appropriate for RNAi-mediated management.

In Chapter 3, I describe my initial work to establish the basic functions of DNMT1 in *B, tabaci.* I show that DNMT1 affects fecundity and offspring viability despite DNA methylation levels remaining the same. I also show that reduction of DNMT1 results in loss of DNMT1 localization in mature oocytes and aberrant morphology of follicular epithelium nuclei. In addition, I show that DNMT1 affects the expression of genes related to cell maintenance and mitotic activity.

Finally, Chapter 4 explores the role of DNMT1 during embryogenesis. I investigated the effects of DNMT1 on embryogenesis in offspring from virgin (all male) and mated (male and female) females. I showed that DNMT1 does not influence embryonic development in a sex-specific manner. Additionally, I show that DNMT1 is required during early embryogenesis for germ rudiment formation. I also established a basic timeline of key stages during embryonic development, which will continue to be a useful tool for whitefly studies.

My combined body of work ultimately does three things: provides another example of how DNMT1 functions during oogenesis and embryogenesis, lays the foundation for further research and development into the use of DNMT1 as a target gene for RNAi-mediated control method, and challenges existing conceptions of the function of DNA methylation and its machinery.

CHAPTER 2

DEBUGGING: STRATEGIES AND CONSIDERATIONS FOR EFFICIENT RNAI-MEDIATED CONTROL OF THE WHITEFLY *BEMISIA TABACI*[1]

[1]Shelby EA, Moss JB, Andreason SA, Simmons AM, Moore AJ, Moore PJ. 2020. *Insects*. 11(11):723. Reprinted here with the permission of the publisher.

2.1 Simple summary

The whitefly *Bemisia tabaci* is a crop pest insect that is difficult to control through commercially available methods. Technology that inhibits gene expression is a promising avenue for controlling whiteflies and other pests. While there are resources available to make this method insect specific, and therefore more effective, it is currently being used in a way that targets insects broadly. This broad approach can cause potential harm to the surrounding environment. Here, we discuss considerations for using gene-silencing technology as a pest management strategy for whiteflies in a way that is specific to this pest, which will address short- and long-term issues of sustainability. We also provide a way of selecting target genes based on their roles in the life history of the insect, which will reduce the potential for unintended negative consequences.

2.2 Abstract

The whitefly *Bemisia tabaci* is a globally important pest that is difficult to control through insecticides, transgenic crops, and natural enemies. Post-transcriptional gene silencing through RNA interference (RNAi) has shown potential as a pest management strategy against *B. tabaci*. While genomic data and other resources are available to create highly effective customizable pest management strategies with RNAi, current applications do not capitalize on species-specific biology. This lack of specificity has the potential to have substantial ecological impacts. Here, we discuss both short- and long-term considerations for sustainable RNAi pest management strategies for *B. tabaci*, focusing on the need for species specificity incorporating both life history and population genetic considerations. We provide a conceptual framework for selecting sublethal target genes

based on their involvement in physiological pathways, which has the greatest potential to ameliorate unintended negative consequences. We suggest that these considerations allow an integrated pest management approach, with fewer negative ecological impacts and reduced likelihood of the evolution of resistant populations.

Keywords: whitefly; *Bemisia tabaci*; RNAi; RNA interference; integrated pest management

2.3 Introduction

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a globally important cryptic species complex that causes billions of U.S. dollars in damages to many crops including vegetable and row crops and therefore presents a major threat to food security (Stansly & Naranjo, 2010; Inoue-Nagata et al., 2016; Czosnek et al., 2017). Over 1000 plant species serve as hosts to *B. tabaci* (Abd-Rabou & Simmons, 2010), and crops that are damaged range from common fruits and vegetables such as cassava (*Manihot esculenta*), squash (*Cucurbita* spp.), and tomato (*Solanum lycopersicum*) to row crops such as cotton (*Gassypium* spp.; Stansly & Naranjo, 2010). Given their mode of feeding, the economic damage caused by *B. tabaci* is extensive because it can be either direct or indirect (Byrne & Bellows, 1991). Like most phytophagous hemipterans, whiteflies feed from the phloem of plants. This feeding behavior affects crops directly in two ways. First, direct feeding can cause adverse plant responses such as chlorotic spots on the leaf surface (Basu, 2019). Damage may result in stunting and can affect the development of reproductive structures of plants, resulting in reduced crop yield in fruits and vegetables (Basu, 2019).

Bemisia tabaci can also cause economic loss without direct impact on the health or vigor of the plant. For example, the diet of sugary sap causes the whiteflies to excrete a sticky substance referred to as honeydew, which coats the plant, results in a buildup of mold, and the economic value of the crops is lowered. This is especially true for cotton and other fibrous plants that are used in textiles (Horowitz et al., 1984; Ellsworth et al., 1999; Hequet et al., 2007). However, the most significant means by which *B. tabaci* causes economic losses in crops is by transmission of plant viruses, including the devastating begomoviruses (Jones, 2003).

Controlling whiteflies has proven difficult. Not only is it difficult to target whiteflies with contact insecticides as they feed from the bottom of leaves, mounting research has made clear that the practice of using pesticides with broad modes of action is costly both economically and biologically, as such methods lead to indiscriminate losses in non-pest insects including beneficial pollinators or natural enemies that feed on *B. tabaci* (Simmona & Jackson, 2000; Simmons & Abd-Rabou, 2005; Simmons & Shaaban, 2011). Moreover, *B. tabaci* has developed resistance to most classes of insecticides (Horowitz et al., 2020). Other pest control strategies, such as the use of *Bacillus thuringiensis* (*Bt*) transgenic crops, have proven ineffective against whiteflies and other sap-sucking insects due to their lack of sensitivity to Cry toxin proteins (Chougule & Bonning, 2012). Other methods, such as biological control measures, have exhibited some success, but often are not able to reduce pest densities to levels that avoid economic losses in a short period of time (Yang et al., 2011). and rely on supplemental insecticide application (Van Driesche et al., 2001; Calvo et al., 2009). Thus, new methods of control are needed, especially those that can be specifically targeted to *B. tabaci*.

RNA interference (RNAi) has emerged as a promising method for pest management. This technology offers a mode of action targeted to specific genes, allowing different physiological systems to be modified to control insect growth, development, or feeding behavior. This gene-silencing mechanism takes advantage of an evolutionarily ancient method used by cells to stabilize the genome against attacks from RNA viruses and foreign genetic elements. RNAi uses exogenous double-stranded RNA (dsRNA) to silence gene expression by co-opting cellular defense mechanisms to target preselected genes (Novina & Sharp, 2004) using dsRNA intermediates, which would not be produced by cells (Zamore, 2001; Geley & Müller, 2004 presents a useful in-depth description of RNAi mechanisms). Upon entry into a cell, dsRNA is cleaved by the enzyme Dicer into short dsRNAs, called short interfering RNAs (siRNAs). The RNA-induced silencing complex (RISC) then degrades the 'sense' strand of the siRNAs, leaving the strand that has a sequence complementary to the target gene, called the 'antisense' strand, to be used for silencing. The antisense strand becomes incorporated into the RISC complex and binds to the complementary messenger RNA (mRNA) based on base pair recognition. The mRNA is then targeted for destruction so that no protein is made. The increased availability of insect genomic and transcriptomic data makes RNAi possible for many insects. This makes RNAi especially useful for B. tabaci because the genomes for three important cryptic species in the species complex (MEAM1/B, MED/Q, and African Cassava whitefly) are published (Chen et al., 2016; Xie et al., 2017; Chen et al., 2019). Moreover, a full genome is not required, only information on target gene sequence (for a thorough discussion, see Geley & Müller, 2004). Because RNAi does not require transformation of the genome, it can be readily applied to non-model species (Whyard et al., 2009).

Researchers have already begun to investigate the viability of deploying RNAi against agricultural pests (Pitino et al., 2011; Zha et al., 2011; Bolognesi et al., 2012). Ongoing discussions on the utility of RNAi as a pest management strategy are centered around perfecting delivery methods such as transgenic plant expression of dsRNA (Thakur et al., 2014; Raza et al., 2016; Ibrahim et al., 2017; Dong et al., 2020), topical applications (Saito, 2005; Gogoi et al., 2017), and endosymbiont-mediated delivery (Whitten et al., 2016) and the ability of the dsRNA construct to silence the target gene and its efficacy to be appropriately evaluated through controls (Baum et al., 2007; Mao et al., 2007). Many reviews have identified the most common pitfalls encountered at each stage of application and offer suggestions on how to improve RNAi efficiency, especially in terms of delivery methods (Burand & Hunter, 2013; Zhang et al., 2017; Grover et al., 2019; Christiaens et al., 2020; Kunte et al., 2020). This body of literature has allowed the scientific community to anticipate possible problems associated with specific RNAi delivery methods applied to *B. tabaci*. However, conceptual frameworks that explore the scope of RNAi's functionality in pure biological terms, considering the appropriate target pathways and desired ecological outcomes, are lacking. Advancing these discussions demands closer attention to knowledge of species specificity, off-target effects, and the possibility that the target species will evolve resistance is lacking (discussed in Zhang et al., 2017). Moreover, the central issue in the use of RNAi as a control strategy, the ideal target, is rarely discussed in terms of lethality without further evaluation of long-term impact, ecological interactions, or sustainability of this approach. This is especially important for complex recalcitrant pests like *B. tabaci*. Here, using *B. tabaci* as a model, we specifically highlight these considerations for the use of RNAi as a part of an integrated pest management strategy.

Our goal is to consider sustainability of long-term use based on the unique features of *B. tabaci* and present a conceptual framework for selecting target genes based on their involvement in specific functional pathways.

2.4 RNAi as a pest management strategy: Need for greater specificity

RNAi has been used for over 20 years to evaluate gene function in insects (Brown et al., 1999; Misquitta & Paterson, 1999; Beye et al., 2002; Schröder, 2003), but only recently has its utility in more commercial applications, including pest management, been considered (Yan et al., 2007; Mamta & Rajam, 2017; Cagliari et al., 2019; Mat Jalaluddin et al., 2019; Vogel et al., 2019). Early studies on coleopteran and lepidopteran pests first demonstrated the potential of RNAi to induce lethality through the knockdown of critical functional genes (Baum et al., 2007; Mao et al., 2007). Since this time, advancements in RNAi technology coupled with the increasing availability of genomic data for non-model insects have expanded the capabilities of researchers to develop more species-specific control mechanisms. Despite this, RNAi for pest management continues to rely on broad stroke approaches and molecular considerations with little attention given to species life history and physiology. Moreover, successful implementation of this new technology remains hindered by familiar pest management issues, including cost efficiency and ecological impacts, that ultimately limit the success of RNAi as a long-term solution. Whiteflies are remarkably well studied among insect pests, and many aspects of successful RNAi implementation in *B. tabaci* have been worked out (Ghanim et al., 2007; Upadhyay et al., 2011; Thakur et al., 2014; Raza et al., 2016; Ibrahim et al., 2017; Luo et al., 2017; Vyas et al., 2017; Eakteiman et al., 2018; Grover et al., 2019; He et al., 2020). According

to overarching consensus, the formula for effective RNAi pest management of *B. tabaci* is to (1) select a target gene that is crucial for survival, (2) use a plant-mediated delivery method, and (3) prevent dsRNA degradation. While this workflow is useful for current implementations of RNAi, it only partially addresses the question, "What is successful application of RNAi in pest control?"

We contend that successful RNAi-mediated control strategies should offer an effective and sustainable alternative or supplement to conventional methods. If true, the present considerations must be expanded to include strategies that minimize ecological impacts and maximize long-term efficacy. This sentiment echoes increasing calls for more holistic approaches to RNAi-mediated pest management (Eakteiman et al., 2018). For the remainder of this review, we focus on three key considerations that have been underrepresented in studies of RNAi in pest management: ecological impact, evolution of resistance, and choice of functional pathway. Each of these considerations relies fundamentally on an understanding of gene function, which we contend must be prioritized for the development of future, species-specific RNAi control strategies.

2.5 Desired outcomes: Lethal versus sublethal effects

Of primary importance for designing and implementing species-specific RNAi control is the careful consideration of desired effects. When it comes to pest management, the desired effect is most often assumed to be lethality (Christiaens & Smagghe, 2014; Thakur et al., 2014; Yu et al., 2014; Li et al., 2015). However, in practice, such blunt-force approaches potentially create more problems than they solve. Below, we expand on these shortcomings and also present the case for substituting sublethal forms of RNAi control as

practically effective, long-term sustainable alternatives to current approaches. In contrast to conventional approaches that manage lethality through dosage administration, we use the term "sublethal" to refer specifically to the magnitude of outcome predicted when knocking down particular gene pathways. In other words, gene targets are selected based on their additive contributions to total fitness as opposed to their necessity for survival.

Short-term considerations: Off-target effects and ecological impact

The first major issue with RNAi applications that result in lethal outcomes lies in the ecological impact of totally eradicating *B. tabaci* from agroecosystems. One possible outcome of this is that a secondary pest species will increase in population density and be more harmful to the cropping system than *B. tabaci* (Pedigo & Rice, 2014). A second potentially harmful effect is the disruption of complex community interactions in which whiteflies are involved outside of agricultural ecosystems (Poelman, 2015; de Rijk et al., 2016). Finally, managing pests through the use of lethal RNAi strategies means that any off-target effects are likely to be especially detrimental (Kulkarni et al., 2006; Baum et al., 2007; Jarosch & Moritz, 2012). One of the ways that RNAi can become a biological hazard is that the dsRNA designed to silence specific target genes aligns, imperfectly or perfectly, to gene regions in another species that share high sequence similarity (Elbashir et al., 2002; Saxena et al., 2003). Such effects become increasingly likely when the targets of RNAi are housekeeping genes with critical functions for survival, as these tend to be highly conserved across arthropods (Wieczorek et al., 2000; Horigane et al., 2007). For example, one study observed that RNAi-induced silencing of core metabolic genes (v-ATPases) in a pest beetle species had significant, unintended effects on survival of non-target beetles

(Baum et al., 2007). Deployed in a mixed agroecosystem, such off-target effects could have devastating consequences.

An alternative to lethal RNAi strategies is to develop a framework that more closely aligns with the goals of integrated pest management. One approach currently being developed for RNAi is based on the use of sublethal methods to control pest populations (Guedes et al., 2017; Mavarro-Roldán & Gemeno, 2017; Müller, 2018; Soares et al., 2020). Together with integrated approaches that target pests only within controlled, locally affected areas, sublethal RNAi approaches offer a promising solution to the issue of unintended ecological consequences arising from the total eradication of whiteflies. A second major advantage of this approach is that it reduces the severity of unintended effects on non-target species (Mallet, 1989; Guedes et al., 2017). This is true because genes that are less crucial for basic cellular functions are also less likely to be strongly conserved. In addition, in the improbable case of perfect sequence alignment, off target effects are less likely to cause mortality. Further, by using sublethal strategies there is the potential to combine RNAi with more traditional and ecologically-based approaches, including the use of insecticides and natural enemies, which may enhance efficacy at reduced cost (Guedes et al., 2017). The use of sublethal strategies could make it possible to utilize parental RNAi for long-term control and could reduce the number of treatment applications. However, this strategy may not be feasible for all gene targets, specifically those that affect reproductive fitness.

Long term considerations: Evolution of resistance

Though the development of resistance is not considered an immediate issue for RNAi-mediated pest control, it remains an important consideration for long-term sustainability (Zhang et al., 2017). The main way that insects become resistant to RNAi appears to be through the acquisition of more efficient dsRNA degradation capabilities (Wynant et al., 2014). However, field insect strains that are resistant to RNAi due to an inability to uptake dsRNA in the gut cells have been reported (Khajuria et al., 2018). Such concerns are pronounced when it comes to managing pests with fast generation times.

Whiteflies exhibit very rapid population growth cycles, which are augmented by their ability to flexibly switch between sexual and asexual modes of reproduction (Byrne & Bellows, 1991). Under optimal conditions, *B. tabaci* females reproduce sexually, resulting in diploid daughters (Byrne & Devonshire, 1996; Figure 2.1A). Because most genetic mutations, including those that confer insecticide resistance, are recessive (Denholm et al., 1996), female offspring must inherit two copies of this new variant to become functionally resistant to RNAi. However, *B. tabaci* is haplodiploid, meaning that females also have the ability to reproduce asexually when suboptimal conditions make it difficult to locate mates (Byrne & Bellows, 1991; Figure 2.1). This results in the production of haploid male offspring, which inherit only a single copy of a new variant and therefore always express this new resistance phenotype. Due to their hemizygous condition, males inheriting mutant (resistant) copies are exposed to selection regardless of dominance or recessiveness (Denholm et al., 1996). The result of this is that can rise to fixation rapidly in populations containing a large proportion of haploid individuals, whereas diploidy delays the evolution of resistance, particularly when beneficial mutations are recessive and

their effects are "shielded" in heterozygous states (Caprio & Hoy, 1995; Denholm et al., 1996; Figure 2.1B). Hence, systems expressing flexible haplodiploidy are already more likely to evolve resistance to RNAi than systems that rely on strict diploidy for their reproduction (Caprio & Hoy, 1995; Denholm et al., 1996). Selecting RNAi targets with sublethal effects, as opposed to lethal effects, offers an intuitive path for slowing the evolution of resistance—by relaxing selection against susceptible individuals (Box 1). In the majority of cases where an insect's pestiferous status can be traced to its high densities, methods of biocontrol that dramatically suppress population growth, rather than inducing mass mortality, are likely to be as effective in mitigating economic damage.

Box 1. A population genetics perspective on the evolution of resistance.

Imagine a hypothetical scenario in which a random mutation creates a genetic variant for resistance (a "resistant allele"), which initially segregates at low frequencies in the population. Two factors—the magnitude of effect of the allele on an individual's phenotype and the strength of natural selection on resulting phenotypes—will determine the speed at which a resistance allele rises to fixation in a population. The efficiency of these dynamics is particularly pronounced in instances where control methods cause lethality, as this mimics a phenomenon referred to in nature as 'hard selection' (Wallace, 1975; Mallet, 1989) Hard selection occurs when alleles are inherited as lethal equivalents, causing immediate death of an organism independent of other selection pressures (Hawkins et al., 2019). The result is strong and efficient purging of non-resistant alleles segregating in the population (Denholm et al., 1995). Consider alternatively a scenario whereby non-resistant alleles do not cause lethality, but instead reduce organismal fitness and gradually suppress population growth. This is referred to as 'soft selection' (Wallace, 1975; Mallet, 1989) and in our example will be applied to any continuous trait imparting sublethal, but severe, effects on fitness. By permitting variation in relative fitness among individuals that carry resistant alleles, soft selection increases the likelihood that some susceptible alleles will be passed on to subsequent generations, thereby relaxing 'hard selective' pressures that drive rapid evolution.

2.6 Selecting suitable targets based on pathway involvement

To prioritize pathways containing genes that are less highly conserved across arthropods and whose knockdown is less likely to cause lethality, an understanding of species-specific gene function is required. Unfortunately, we do not always have gene function defined across species. Overwhelmingly, given the status of *Drosophila melanogaster* as a long-standing genetic model organism, dipteran model systems serve as the standard from which entomologists have come to understand gene function in insects (Bellés, 2009). Even though genomic and transcriptomic resources are increasingly available for common hemipteran pests, including whiteflies (Chen et al., 2016; Xie et al., 2017; Chen et al., 2019), the function of many insect genes are rarely assigned based on functional genomics or other functional studies. Rather, many of the assigned gene functions have been extrapolated from laboratory experiments on *D. melanogaster* and/or inferred from sequence similarity with *Drosophila* homologues (Watson et al., 2005; Costello et al., 2009). These approaches are imperfect, as hemipteran pests, including whiteflies, exhibit distinct life history strategies and physiology. Thus, current and ongoing functional genomic studies will provide invaluable insight into selecting genes to silence. A further reason that selecting RNAi targets in pathways of known functions is a desirable long-term option over reliance on annotated functions extrapolated from *Drosophila* is that this would allow for more precise scientific monitoring and troubleshooting of RNAi behavior.

Categories of sublethal target pathways include those involved in gametogenesis, immunity, movement, mating, and appetite stimulation. Targeting genes from pathways involved in the detoxification of plant defenses has already shown to be successful at

reducing, but not eradicating, *B. tabaci* populations (Eakteiman et al., 2018). Table 2.1 provides examples of potential targets. *For B. tabaci*, genes related to mating behavior may not be effective because of their ability to reproduce parthenogenically. More promising are genes targeting gametogenesis and immunity. Knocking out reproductive capacity is attractive because *B. tabaci* reproduction can be knocked out at several stages. Potential genes include those involved in gamete viability such as *Boule*, *Vasa*, or *DNA methyltransferase 1* (*Dnmt1*; Gustafson & Wessel, 2010; Sekiné et al., 2015; Bewick et al., 2019; Amukamara et al., 2020). The latter suggestion is a good example of the importance of understanding specific gene function. Conventionally, *Dnmt1* is expected to maintain methylation in the genome. However, methylation in insects does not appear to be universal, and some species such as the genetic model species *D. melanogaster* and *Tribolium confusum* lack *Dnmt1* altogether (Bewick et al., 2016) Yet in *Oncopeltus fasciatus*, a hemipteran, co-authors have shown through RNAi knockdown that *Dnmt1* has an essential function in gamete production in both males and females and that the silencing of *Dnmt1* results in sterility without causing mortality or disrupting somatic tissue (Bewick et al., 2019; Amukamara et al., 2020). The *Dnmt1* gene sequence has been identified in *B. tabaci* and successfully knocked down using RNAi (Dai et al., 2017).

Another possible sublethal target is immunity. Targeting immunity genes has the two-fold benefit of reducing fitness and increasing efficiency of the dsRNA construct. The immune system barriers that evolved to protect *B. tabaci* from infection also have the capability to degrade the dsRNA before it can be processed by the endogenous RNAi machinery. In many cases, the dsRNA is ingested and subsequently degraded by nucleases in the saliva or the gut (Christiaens & Smagghe, 2018). This phenomenon has been

observed in many insects and contributes to variation in RNAi efficiency seen between and among species (Terenius et al., 2011; Scott et al., 2013; Wang et al., 2016) It has been demonstrated in *B. tabaci*, as well as other insect orders, that dsRNA-mediated silencing of digestive nucleases enhances the efficiency of silencing target genes (Luo et al., 2017; Guan et al., 2018; Prentice et al., 2019; Tayler et al., 2019) .

2.7 Conclusions

The remarkable diversity of pest management strategies is matched only by the ability of insects to overcome them. RNAi strategies that take into consideration life history and population genetics are likely to be valuable control strategies. Success in *B. tabaci* control will depend not only on building on our knowledge of the success and failures of current approaches, but also on how well we address long-term issues. Using RNAi to resolve these challenges offers a unique opportunity to explore these novel aspects of *B. tabaci*, including evolution of resistance and reproductive biology, while also managing their economic damage. It also makes it possible to address other issues such as application effects and cost issues, which are crucial factors for the development of practical biopesticides. For the best long-term outcomes, future RNAi applications using *B. tabaci* should consider selecting non-lethal target genes that confer continuous traits such as fecundity. Further developing RNAi for use as a sustainable pest management strategy will require extensive collaboration between IPM specialists, laboratory scientists, and public policy makers.

2.8 Acknowledgements and funding

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Table 2.1. Examples of potential sublethal genes as targets of RNAi pest control.

Function	Potential Genes	Citations
Appetite stimulation/feeding	*For (foraging)* *Anox (anorexia)*	(Tarés et al., 2013) (Ryuda et al., 2011; Majerowicz et al., 2016)
Immunity/detoxification	*Def (defensin)* *DsRNase* *GST (glutathione S-transferase)*	(Wang et al., 2017) (Luo et al., 2017) (Eakteiman et al., 2018)
Mating/reproduction	*Fru (fruitless)* *Dsf (dissatisfied)* *Croaker* *Per (period)*	(Clynen et al., 2011) (Finley et al., 1997) (Yokokura et al., 1995) (Wise et al., 2002)
Gametogenesis	*Boule* *Vasa* *Dnmt1 (DNA methyltransferase 1)*	(Sekiné et al., 2015) (Gustafson & Wessel, 2010) (Amukamara et al., 2020; Bewick et al., 2016)

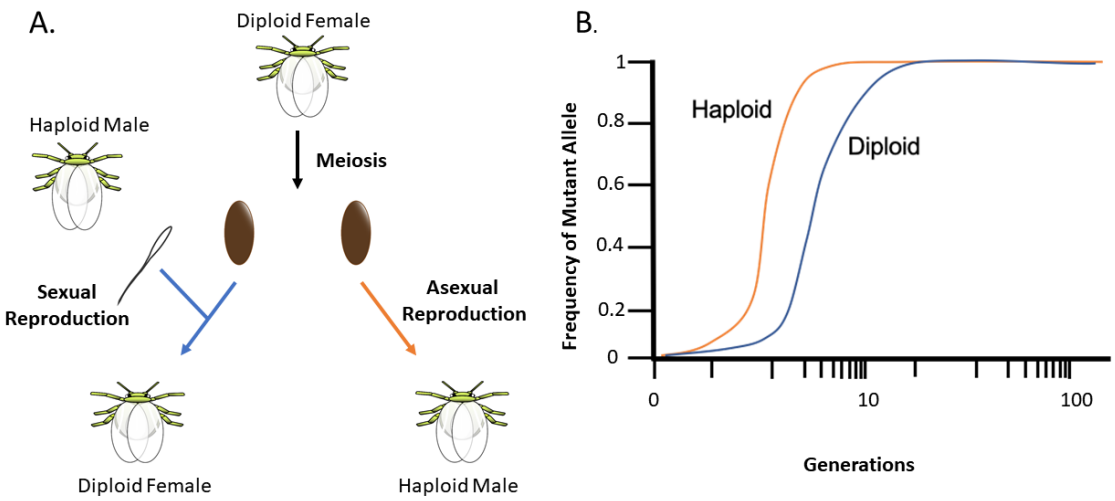

Figure 2.1. Arrhenotoky in *Bemisia tabaci* and fixation of alleles. (A) Sexual and asexual reproduction. Sexual reproduction results in diploid female offspring while asexual reproduction results in haploid male offspring. (B) Hypothetical rate of fixation of mutation alleles haploid vs. diploid organisms. Rate of mutant allele fixation occurs faster in haploid populations (Modeled from Mallet, 1989).

CHAPTER 3

THE ROLE OF DNMT1 IN OOCYTE DEVELOPMENT[2]

[2]Shelby EA, McKinney EC, Cunningham CB, Simmons AM, Moore AJ, Moore PJ. 2023. *J. Insect Physiol.* 147:104507. Reprinted here with the permission of the publisher.

3.1 Abstract

The whitefly *Bemisia tabaci* is a globally important crop pest that is difficult to manage through current commercially available methods. While RNA interference (RNAi) is a promising strategy for managing this pest, effective target genes remain unclear. We suggest *DNA methyltransferase 1* (*Dnmt1*) as a potential target gene due to its effect on fecundity in females in other taxa of insects. We investigated the role of *Dnmt1* in *B. tabaci* using RNAi and immunohistochemistry to confirm its potential conserved function in insect reproduction, which will define its usefulness as a target gene. Using RNAi to downregulate *Dnmt1* in female *B. tabaci*, we show that *Dnmt1* indeed has a conserved role in reproduction, as knockdown interfered with oocyte development. Females in which *Dnmt1* was knocked down had greatly reduced fecundity and fertility; this supports *Dnmt1* as a suitable target gene for RNAi-mediated pest management of *B. tabaci*.

Keywords: *Dnmt1*; Reproduction; RNA interference; Whitefly; *Bemisia tabaci*; Pest management

3.2 Introduction

The whitefly *Bemisia tabaci* is among the top ranked insect pests worldwide. Because this globally-important cryptic species complex causes billions of U.S. dollars in damages to food crops through feeding or transmitting diseases, it presents a major threat to food security (Stansly et al., 2010; Inoue-Nagata et al., 2016; Czosnek et al., 2017). Whiteflies also pose a threat to non-food crops such as cotton and ornamental plants (Stansly et al., 2010). Moreover, these threats are heightened by the difficulty in managing

this pest as a result of its biology. Whiteflies have developed resistance to most classes of insecticides (Horowitz et al., 2020). The industry standard for treating whiteflies is neonicotinoids (Nauen & Denholm, 2005), which is problematic because they affect surrounding pollinator populations (Lu, Hung, & Cheng, 2020). Other, non-chemical, methods of pest management have proven to be less effective against whiteflies due to their feeding behavior and physiology (Chougule & Bonning, 2012). Thus, RNA interference (RNAi) has gained support as a viable defense strategy against whiteflies (Pitino et al., 2011; Zha et al., 2011; Bolognesi et al., 2012). However, this choice for pest management poses potential ecological problems as the usual target genes of choice are highly conserved across the insect taxa, which could lead to indiscriminate losses in non-pest insects in addition to whiteflies (Shelby et al., 2020). To minimize adverse ecological impacts and maximize efficiency of RNAi as a pest management strategy, there needs to be a fundamental understanding of the target genes (Shelby et al., 2020).

DNA methyltransferase 1 (*Dnmt1*) may be a suitable target gene for RNAi-mediated management of *B. tabaci*. *Dnmt1* is a highly conserved gene of Bilateria (Glastad et al., 2011) that is responsible for maintaining DNA methylation patterns after cell division in plants and mammals (Goll et al., 2005; Goll et al., 2006; Schmitz et al., 2019). While the general function of *Dnmt1* and its importance for methylation in insects is unclear (reviewed in Duncan et al., 2022), *Dnmt1* consistently appears to be involved in reproduction (Zweir et al., 2011; Kay et al., 2018; Schulz et al., 2018; Bewick et al., 2019; Amukamara et al., 2020; Washington et al., 2021). For example, *Dnmt1* is required for oogenesis of the hemipteran *Oncopeltus fasciatus,* and knockdown of *Dnmt1* results in reduced fecundity in this species (Bewick et al., 2019; Amukamara et al., 2020). However,

unexpectedly, this loss of *Dnmt1* does not result in reduced survivorship or somatic defects (Bewick et al., 2019; Amukamara et al., 2020). If, as in *O. fasciatus*, *Dnmt1* affects female *B. tabaci* reproduction without affecting survivorship, *Dnmt1* would be both a safer target gene for management of *B. tabaci* and could be used in combination with biocontrol methods that are currently used for *B. tabaci* management (Kheirodin et al., 2020). Reducing population growth without affecting survivorship would also reduce unintended detrimental effects towards off-target species. The utility of *Dnmt1* as a target gene is even greater when coupled with the RNAi machinery used for gene silencing. Even though the presence of a *Dnmt1* gene is highly conserved, sequence homology is likely to be too low to be an ecological risk because even a single nucleotide mismatch can disrupt the RNAi machinery needed for gene silencing (Mocellin & Provenzano, 2004).

One caveat regarding the use of *Dnmt1* as a target gene is that although *Dnmt1* clearly affects reproduction in many insect taxa (Bewick et al., 2019; Gegner et al., 2019; Amukamara et al., 2020; Washington et al., 2021; Arsala et al., 2022), it is unclear *how* it affects reproduction. There are currently two hypotheses as to which parts of reproduction *Dnmt1* influences (Amukamara et al., 2020; Washington et al., 2021). The first is that *Dnmt1* is required for progression of gametogonia into gametocytes. This hypothesis is based on the observation that loss of *Dnmt1* prevents both oogonia and spermatogonia from properly advancing through meiosis, which results in reduced or absent production of early oocytes and spermatocytes (Amukamara et al., 2020; Washington et al., 2021). The alternative hypothesis is that *Dnmt1* is needed for germ cell viability, and loss of *Dnmt1* affects gametocyte stability broadly through an unknown mechanism (Amukamara et al., 2020; Washington et al., 2021). Considering the diversity of insect reproductive modes and

processes, knowing the developmental pathways through which *Dnmt1* acts allows the scientific community to better understand the extent of *Dnmt1* functional conservation. In the case of pest management strategies, understanding the mechanisms underpinning *Dnmt1'*s role in reproduction allows for better prediction of which off-target species could also be affected.

In this study, we demonstrate the viability of *Dnmt1* as a target gene for RNAi-mediated control of whiteflies after investigating its role in *B. tabaci* oogenesis. Specifically, our objective was to test the hypothesis that, as with *O. fasciatus*, *Dnmt1* is required for successful oogenesis. We first used RNAi to knockdown expression of *Dnmt1* and to evaluate its major phenotypic effects on female *B. tabaci* with survival and fecundity assays. Then, we used immunohistochemistry and microscopy to visualize ovarian and oocyte structure to document oocyte development *Dnmt1* perturbations after treatment. Finally, we examined gene expression of several candidate pathways through which *Dnmt1* may function.

3.3 Materials and methods

Insect rearing

All experimental individuals were derived from colonies of laboratory-reared *B. tabaci.* The colonies were started from populations of MEAM1 *B. tabaci* collected from a cotton field site in Tift County, Georgia in 2018 (McKenzie et al., 2020). Our colonies were maintained on collard (*Brassica oleracea*) plants grown in individual pots maintained and grown to a minimum height of 15 cm. Both whiteflies and collards were maintained at 26ºC with a 14:10 hour light: dark photoperiod in 28 cm x 28cm x 51 cm aluminum insect

cages (Bioquip Products, Rancho Dominguez, CA, USA). These conditions were constant throughout the bioassays.

RNA interference (RNAi)

We prepared DNA templates of *Dnmt1* and *eGFP* (the control treatment) using PCR amplification with gene-specific primers and 500 ng/µl RNA (Table 3.1). We synthesized sense and antisense RNA in a single reaction using the Ambion MEGAscript RNAi kit (ThermoFischer Sci, Waltham, MA, USA) following the manufacturer's instructions. Following extraction and ethanol precipitation, we aliquoted the double-stranded RNA (dsRNA) and stored at -80ºC.

To ensure the females were unmated, newly-eclosed females were removed from nymph colonies within 24 hours of eclosion. At the time of RNAi treatment, females were 3-6 days post eclosion. We treated adult female *B. tabaci* with dsRNA using an artificial feeding mechanism (Figure 3.1). Briefly, we prepared a dsRNA solution with either *dsDnmt1* or *dseGFP* using a 10% w/v sucrose solution in RNase-free water. We added green food coloring (McCormick & Company, Baltimore, MD, USA) to the feeding solution to facilitate visual confirmation of ingestion of the solution. The feeding set-up was based on a set-up used for artificial blood feeding (Harrison et al., 2021). We pipetted the feeding solution into the cut-off caps of 1.7 ml microfuge tubes (ThermoFischer Sci, Waltham, MA, USA), to which we added 200 µl of feeding solution, resulting in 100 µg of dsRNA per treatment group. Parafilm® M (Millipore Sigma P7543) was stretched over these caps to produce small capacity membrane feeders. One cap was placed on the mesh top of an acetate tube, membrane-side down; 75-100 female whiteflies were treated in each

tube. We then wrapped the tube with black construction paper around the sides to promote movement to the top of the tube, thus promoting feeding behavior. We allowed females to feed in the tubes for 24 hours. We confirmed the females had fed by observing green food coloring in the abdominal region. Only confirmed females were used in subsequent experiments. After the 24 hours in the artificial feeding apparatus, we either froze the females in liquid nitrogen for use in RNA extractions (n = 8 & 11) or DNA extractions (n = 3 each) or placed as part of a biological replicate in an insect cage (as described above; n = 10 for each treatment) with a 15 cm tall collard plant for use in life history or microscopy experiments.

Survival, fecundity, and viability assays

Survival assay

After RNAi treatment, we aspirated and moved each biological replicate (n = 10 per treatment) of adult female whiteflies into insect cages (as described above) with a 15 cm tall collard plant for feeding and oviposition. Each biological replicate contained 75 – 100 treated females. Because female *B. tabaci* reproduce arrhenotokously, males were not needed to produce offspring. We monitored control and experimental females every 24 hours for number of survivors. We recorded the number of surviving females each day for a total of 5 days.

Fecundity assays

For fecundity assays, we replaced the plants daily and recorded the total number of eggs laid for five days. To account for the variation in the number of living females between plants and across the experiment, we determined the number of eggs per female by dividing

the number of eggs per day by the number of living females per biological replicate (n = 10 biological replicates per treatment). Each replicate group originally contained 75-100 females.

Viability assays

For embryo viability assays, we recorded the number of eggs from the fecundity assay that hatch from both treatments (n = 3 per treatment haphazardly chosen from the 10 replicates used in survival and fecundity assays). As a follow-up experiment, we investigated whether the proportion of non-viable eggs from individual clutches laid by *dsDnmt1*-treated females changed over time. Following RNAi treatment with *dsDnmt1* or *dseGFP*, we placed 30 females onto each collard plant. For five days, we quarantined egg clutches using a clip cage and monitored them daily for development (i.e., change in egg color). We recorded the number of eggs that developed within each clutch.

Microscopy and immunohistochemistry

We dissected adult females from both treatments (*dsDnmt1* and *dseGFP*) three days after treatment to visualize ovarian and oocyte structure (Figure 3.2). At the time of the dissection, the females were 7-10 days post eclosion. After anaesthetization on ice, we removed the ovarioles by tearing open the abdomen in a drop of 10% BSA in 1X PBS. We fixed the ovaries and stained them following our previous protocol (Amukamara et al. 2020). Briefly, we transferred ovarioles to 4% paraformaldehyde in PBT for fixation. Following fixation, we determined DNMT1 protein localization by incubated the ovarioles in a commercial anti-rabbit DNMT1 primary antibody to visualize DNMT1 (Abcam, Cambridge, UK). We used an Alexa Fluor goat-anti-rabbit 647 secondary antibody

(Thermofisher Scientific, Waltham, MA, USA). We visualized nucleic acids by adding 1 μL 0.5 μg/mL, DAPI to the final wash buffer. We mounted the stained ovarioles with Mowiol 4-88 mounting medium (Sigma-Aldrich, St. Louis, MO, USA). We determined the developmental stage of each oocyte based on the descriptions in Guo et al. (2010). We recorded the number of oocytes from each developmental stage for both treatments. We imaged the ovarioles and oocytes with a Zeiss LSM 710 Confocal Microscope (Zeiss) at the University of Georgia Biomedical Microscope core. All confocal images were falsely colored.

We visualized the trophocyte cells in each tropharium (n = 20 tropharium for *dseGFP* and n = 21 tropharium for *dsDnmt1*). Each tropharium was z-stack imaged at 100X magnification to create a maximum intensity projection image and we counted the number of trophocyte nuclei (red dots in Figure 3.2B). Next, we imaged each vitellarium at 20X magnification to create maximum intensity projection images and measured the number of follicular cell nuclei per mature oocyte (blue squares in Figure 3.2B) and the area of each follicular cell nuclei (n = 1190 for *dseGFP* and n = 827 for *dsDnmt1*). All measurements were done using ImageJ (Abràmoff et al., 2004).

Quantification of DNA methylation

We compared the percent of DNA methylation between our control (*dseGFP*) and treatment (*dsDnmt1*) samples using Fast^{m}C (Bewick et al., 2016). Briefly, DNA was extracted from 3 biological replicates, containing 25 females each, for each treatment using a Qiagen Allprep DNA/RNA Mini Kit (Qiagen, Venlo, the Netherlands) following the

manufacturer's protocol. Whole genome bisulfite sequencing (WGBS) libraries were prepared and sequenced by Novogene (www.Novogene.com). The percent DNA methylation level was calculated by dividing the total number of methylated CpG sites by the total number of CpG sites (Schultz et al. 2012).

RNA extraction and qRT-PCR

To confirm *Dnmt1* knockdown, we extracted total RNA from whole bodies of adult female whiteflies from either *dseGFP* (n = 8 biological replicates) or *dsDnmt1* (n = 11 biological replicates). Each biological replicate contained 25 females. We used the Qiagen RNA Easy kit with Qiazol (Qiagen, Venlo, The Netherlands) following the manufacturer's protocol and a BeadBug™ Microtube Homogenizer (Benchmark Scientific, Sayreville, NJ, USA) to extract RNA. Complementary DNA (cDNA) was synthesized from 500 ng RNA with qScript cDNA Super-Mix (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's protocol. We quantified expression levels of *Dnmt1* using quantitative real-time PCR (qRT-PCR). Our primers were designed using the whitefly genome as a reference (Chen et al., 2016; Table 3.1). GAPDH was used as our endogenous reference gene. All samples were run with 3 technical replicates using 10 µl reactions on the Roche LightCycler 480 with SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN, USA). We performed primer efficiency assays, genomic contamination testing, and endogenous reference gene selection as described in Cunningham et al. (2014).

To examine the effects of *Dnmt1* expression on genes involved in cell function, we quantified expression levels of *Dnmt1, Inhibitor of apoptosis (Iap), Signal transducer and activator of transcription* (*Stat), Cell division cycle 20* (*Cdc20), Cell division cycle 25*

(*Cdc25)*, and *SPO11 initiator of meiotic double stranded breaks* (*Spo11)* using qRT-PCR as described above. We used the same samples as the previous experiment (n = 8 for *dseGFP* and n = 11 for *dsDnmt1* with 25 pooled females in each). We designed the primers using the whitefly genome as a reference (Chen et al., 2016; Table 3.1).

Statistical analyses

To test for differences in longevity relative to treatment, we used the log-rank test in the *survival* and *survminer* packages in RStudio (Kassambara, 2017). We used an unequal variance t-test to compare the mean number of eggs per female for each treatment. We then used a repeated measures ANOVA to determine significant difference in mean number of eggs between and within treatments. To compare the mean number of eggs that hatched in each treatment, we used an unequal variance t-test. We investigated whether the proportion of non-viable eggs from individual clutches laid by *dsDnmt1*-treated females changed over time, we used a repeated measures ANOVA.We used a Chi-square test to determine the association between presence/absence of oocyte nuclei (large black dot in maturing oocyte in Figure 3.2B) and treatment. We used a t-test to compare the mean number of trophocytes and the mean follicular cell nuclei area between the treatments. We used the $\Delta\Delta C_T$ method to compare differences in gene expression using the dseGFP treatment as our comparison group (Livak & Schmittgen, 2001). We then used an unequal variance t-test to compare mean expression between treatments. For all data analyses, we used Base R in RStudio (RStudio Team, 2020). We used the ggplot package for data visualization (Wickman, 2016).

3.4 Results

Dnmt1 knockdown reduces fecundity and viability but not survivorship in female *B. tabaci.*

Following dsRNA feeding, *Dnmt1* expression was reduced in adult females treated with *dsDnmt1* (t_{17} = -2.434, p = 0.026; Figure 3.3). Reduced expression of *Dnmt1* did not affect survivorship; there was no difference in survivorship between *dsDnmt1*-treated females and *dseGFP*-treated females (Log-rank, p = 0.166; Figure 3.4A). There were no observable differences in female morphology or behavior following consumption of dsRNA. Females from both treatments readily fed on host plants following treatment, suggesting no effect on feeding behavior.

Females treated with *dsDnmt1* produced statistically significantly fewer eggs (t_{18} = -10.11, $p < 0.001$; Figure 3.4B). The *dsDnmt1* females laid an average of 3 ± 1.2 eggs per female whereas the *dseGFP* females laid an average of 7 ± 0.8 eggs per female. The number of eggs produced over time did not increase or decrease within either treatment ($F_{4,88}$ = 1.891, p = 0.119; Figure 3.4C). Again, there was also a statistically significant effect of treatment on the number of eggs produced between treatments when accounting for time ($F_{1,88}$ = 182.442, $p < 0.001$; Figure 3.4C).

Dnmt1 knockdown reduced egg viability. Only 24.5 ± 32.0 % of total eggs from the *dsDnmt1* treatment hatched compared to the 83.3 ± 19.1% of total eggs hatched from the *dseGFP* treatment (t_4 = -9.0363, p = 0.001). Clutches laid by *dsDnmt1*-treated females had a mix of both viable and non-viable eggs (Figure 3.5A). At the level of individual clutches, which are produced by a single female, only 45.0 ± 24.9 % of eggs hatched from

dsDnmt1-treated females compared to the 99.0 ± 2.53% of eggs hatched from *dseGFP*-treated females (t_{178} = -20.36, $p < 0.0001$).

Reduction of *Dnmt1* expression results in oocyte abnormalities

At the time of dissection (7-10 days post eclosion), most of the oocytes were in the fourth and final stage of development (46 ± 19.0 % for *dsDnmt1* and 69 ± 21 % for *dseGFP*); 30 ± 12% of *dsDnmt1* and 16 ±12% of *dseGFP* oocytes were in the third stage of development, and 14 ± 11% of *dsDnmt1* and 10 ± 11% of *dseGFP* were in the second stage of development. There was no statistical difference in the frequency of oocytes at a certain stage of development between the two treatments (X^2 = 0.10175, d.f. = 3, p = 0.992). There was also no visible difference in ovariole size, shape, or color when comparing ovarioles of the same stage between treatments.

Reduction of *Dnmt1* did not produce phenotypic effects of the tropharium of the ovariole. In both treatments, the trophocyte nuclei were visible, as well as the nucleus of the segregating oocyte (Figures 3.6A, 3.6C, 3.6D, 3.6F). There was no statistically significant difference in the mean number of trophocyte nuclei between the treatments (t_{40} = 0.17501 , p = 0.862; Figure 3.6G). In both treatments, DNMT1 was detectable in the tropharium, and was localized in the cytoplasm around the nuclei (Figures 3.6B, 3.6C, 3.6E, 3.6F).

The vitellarium was affected by the knockdown of *Dnmt1*. While DNMT1 was detectable throughout the vitellarium of both treatments, the protein was not localized in the nucleus of the maturing oocytes of *dsDnmt1*-treated individuals (Figures 3.7B, 3.7C). The absence of nuclear localization was observed in 17 out of 21 maturing oocytes. This

is statistically significantly different from what was seen with the maturing oocytes of *dseGFP* -treated individuals where DNMT1 clearly marked the maturing oocyte nucleus in 19 out of 20 observations (X^2 = 26.779, d.f. = 1, p < 0.001; Figures 3.7E, 3.7F white arrowhead). Reduction of *Dnmt1* also affected the follicular cells. Follicular cells from the *dseGFP* treatment had round nuclei with a mean area of 59.9 ± 11.7 μm^2 (Figures 3.8B, 3.8C). This was significantly different from those from the *dsDnmt1* treatment, which had an irregular shape and a mean area of 113.4 ± 23.9 μm^2 (t_{38} = 16.216, p < 0.001; Figures 3.8A, 3.8C).

DNA methylation levels were not reduced by *Dnmt1* reduction.

While expression of *Dnmt1* was reduced in our *dsDnmt1*-treated samples, there was no reduction in percent of methylated CpG DNA compared to controls. Genomic DNA from both *dseGFP*-treated and *dsDnmt1*-treated females had approximately 5% CpG methylation (95% CI: 0-24%; Figure 3.9).

Reduction of *Dnmt1* affects expression of genes related to cell maintenance and mitosis.

While we did not observe morphological evidence of apoptosis in *dsDnmt1*-treated females, expression levels for genes involved in the inhibition of apoptosis (*Iap* and *Stat*) were statistically significantly lower in *dsDnmt1*-treated females (*Iap*: t_{17} = -2.4122, p = 0.027; *Stat*: t_{17} = -2.3818, p = 0.0158; Figure 3.10A). Similarly, reduction of *Dnmt1* significantly reduced the expression of *Cdc20* (t_{17} = -3.6438, p = 0.002), which plays a role in mitosis (Figure 3.10B). *Cdc25*, another gene involved in mitosis, had reduce -but

not significantly reduced- expression in *dsDnmt1*-treated females (t_{17} = -2.0006, p = 0.063; Fig. 10B). Expression of *Spo11,* a gene involved in meiosis, was not significantly affected (t_{17} = -1.1289, p = 0.275; Figure 3.10B).

3.5 Discussion

RNAi-mediated pest control continues to be proposed for hard-to-manage species like *B. tabaci* through various methods. However, the best choice of target genes remains to be settled. Most research has focused on using highly lethal genes (reviewed in Shelby et al., 2020 and Hunter & Wintermantel, 2021; Jain et al., 2022). Additionally, little information has been gathered about mode of action of these genes in target species. In this study, we help bridge the gap between pest management and basic biology by assessing the potential use of a conserved, non-lethal target gene and documenting the effects of the gene using RNAi.

Knockdown of *Dnmt1* reduced fecundity (the number of eggs) and viability (percent of viable eggs) without impacting survivorship. In terms of pest management, slowing down population growth instead of wiping out the population is favored because it allows for biocontrol methods, like beneficial predators, to be more effective (Schmidt et al., 2020). It also lessens the effects in off-target species (Schmidt et al., 2020). In the case of *Dnmt1*, even though it is a highly conserved gene, reduction of *Dnmt1* in many taxa does not lead to detrimental effects. For example, reduction of *Dnmt1* in *O. fasciatus* does not lead to decreased survivorship (Bewick et al., 2019; Amukamara et al., 2020; Washington et al., 2021). Based on the effects of our knockdown studies, we suggest

Dnmt1 is a viable target gene for *B. tabaci* control that is compatible with biocontrol strategies.

Our work also has implications for the understanding of the pleiotropic role of *Dnmt1* in insects. Our results confirm that *Dnmt1* has a conserved role for reproduction. Knockdown of *Dnmt1* in *B. tabaci* phenocopies what is seen in *O. fasciatus*: reduction of *Dnmt1* affects oocytes, but not trophic cells. This suggests *Dnmt1* function might be specific to oocytes. DNMT1 is not localized in the trophocyte nucleus. The lack of nuclear localization is unexpected considering that DNMT1 is a DNA methyltransferase, and therefore, would need to have access to DNA. Unlike *O. fasciatus*, we did not see a reduction in the number of primary oocytes, which could be interpreted as *Dnmt1* not playing a role in meiosis in *B. tabaci.* Expression of *Spo11*, a gene responsible for initiating meiosis, was not significantly affected by reduction of *Dnmt1*. However, the difference we see between these two hemipterans could also be due to the timing of our experiment (Amukamara et al., 2020). By the time we treated the *B. tabaci* females, their oocytes were already farther in development, having already progressed through meiosis prior treatment. Our results suggest that *Dnmt1* may be functionally conserved in many taxa because it targets oocytes. This knowledge can be leveraged for pest-management as it allows one to predict which processes *Dnmt1* may affect in off-target species.

There are two hypotheses as to how *Dnmt1* controls reproduction: by ensuring progression through meiosis or by providing oocyte stability (Bewick et al., 2019; Amukamara et al., 2020; Washington et al., 2021). This study suggests *Dnmt1* plays a role in oocyte stability. Although we did not observe phenotypic differences when looking at the tropharium from either treatments, there were drastic differences between the two

treatments in the vitellarium. The most striking difference was the localization (or lack thereof) of DNMT1 into the maturing oocyte nucleus. This could be because DNMT1 proteins were unstable, breaking down and were not replaced following RNAi knockdown. Alternatively, DNMT1 proteins were stable, but unable to move into the nucleus. DNMT1 is known to control cytosine methylation in plants and mammals (Schmitz et al., 2019), so localization to the nucleus is predicted if the same role is performed in insects. Another difference that points to oocyte stability is the abnormal follicular cell nuclei. Follicular cells are needed to support oocyte maturation, and loss of of the follicular epithelium is likely detrimental. Indeed, reduced expression of *Iap* and *Stat*, which inhibit apoptosis, suggest that apoptosis may be triggered when *Dnmt1* is reduced.

While our results clarify the essential role of DNMT1 in oocyte development and viability, our experiments did not specifically address whether the role of DNMT1 is tied to its role as a methyltransferase or in a non-canonical role. While *B. tabaci* has the DNA methylation toolkit, DNA methylation has not been documented in this species using the gold standard of whole genome bisulfite sequencing (Dai et al., 2017). Here, we show that *B. tabaci* DNA does contain CpG methylation in their genome but DNA methylation levels did not change between *dseGFP*-treated females and *dsDnmt1*-treated females. However, given the timing between treatment with the dsRNA and sampling, we predicted that we would not see any changes in DNA methylation levels in the somatic and reproductive cells, as the majority of cells will not have synthesized DNA and divided during the course of the experiment (Wick & Bonhag, 1955) . This prediction is upheld. Thus, this study adds to the growing body of literature in which DNA methylation and the action of DNMT1 is uncoupled (Zwier et al., 2011; Schulz et al., 2018; Bewick et al., 2019; Amukamara et al.,

2020; Washington et al., 2021; Duncan & Cunningham, 2022) . For example, changes in DNA methylation patterns and reproductive phenotype, like what is seen here, are uncoupled from specific changes in transcriptional patterns in the related *O. fasciatus* (Bewick et al., 2019). This result is mirrored in our expression data where we see changes in some, but not all examined genes, despite no change in percent DNA methylation. While we cannot rule out small, targeted modifications of DNA methylation, this is unlikely given that we do not expect cells to have divided over the course of the experiment.

The strongest evidence strengthening the argument that DNMT1 may also play a role outside of simple maintenance of methylation is that the reproductive phenotype of *Dnmt1* knockdown manifests even in species that do not have CpG DNA methylation. The most striking example of this phenomenon is how loss of *Dnmt1* in *Tribolium casteneum*, an insect that lacks detectable levels of DNA methylation, only affects reproductive tissues (Zemach et al., 2010). The current data indicates that DNA methylation can be lost without a loss of somatic phenotype, making it likely that the functional role of *Dnmt1* in reproduction and its role in DNA methylation are not mutually exclusive (Amukamara et al., 2020). This concept becomes more apparent when looking across the insect tree of life and seeing the variation in the presence/absence of *Dnmt1* as well as gene duplications. Variation of DNA methyltransferase toolkits do not correspond to DNA methylation levels (Bewick et al., 2019). If the only functional role of *Dnmt1* is to maintain DNA methylation patterns, why does it produce the same reproductive phenotype, regardless of the insects DNA methylation levels? Further studies must be done to understand the mechanisms and developmental pathways through which *Dnmt1* controls reproduction. Regardless of

whether this reproductive phenotype in *B. tabaci* stems from changes in DNA methylation or not, these results provide a better understanding of *Dnmt1* for safer pest management.

3.6 Conclusion

We demonstrated that *Dnmt1* reduces fecundity and viability of female *B. tabaci*. Mechanistically, it affects the development of the maturing oocyte and its follicular cells. Based on this work, we suggest that *Dnmt1* plays a required role for oocyte stability. Because oogenesis has diverse requirements, we can leverage these differences to better predict off-target species. For these reasons, we suggest that *Dnmt1* is a candidate target gene for RNAi-mediated pest control of *B. tabaci* and possibly other whitefly pests.

3.7 Acknowledgements and Funding

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Table 3.1. Primer sequences used for qRT-PCR and dsRNA synthesis. T7 primer sequences are shown in lower case letter.

Gene	Sense Primer	Anti-sense Primer
dsRNA synthesis primers		
Dnmt1	taatacgactcactatagggaga + TCAATGATCATGATGAAAGGCCGCA	taatacgactcactatagggaga + TGTCAGTGCTGACATTCCACACGGA
eGFP	taatacgactcactatagggaga + CGAATTCACTAGTGATTTTACTTG	taatacgactcactatagggaga + GCGGGAATTCGATTTGACC
qRT-PCR primers		
Dnmt1	CTCGATAATGCCATCCGTAGTT	CACTTTCCTCTGGGACATCTTT
Cdc20	TCAGCGAGTCGCATCATTATCA	GGCTACTTGGTGTTCCCTTT
Cdc25	GCTTCACCAAATACCAACCTAC	GTCACCAATCAGCTCTTCTTTC
Spo11	CTGGGAAGGGATTTCCTGATTT	GGATCAGCATCGGCAATCATA
Iap	GGAGCTGTTCCCTCAGAAATAA	TCCATCATCATTGCCACGTAA
Stat	GGACAAGGCGTAAATGCTAATG	CAACTCGACGAACACACTCT
Gapdh	CAACGGATTTGGCCGTATTG	CCGTGGGTGGAATCATACTT

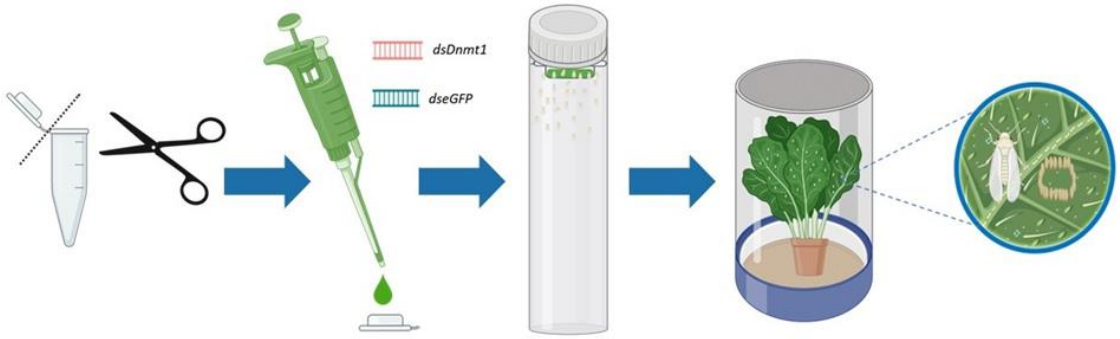

Figure 3.1. Schematic of the dsRNA artificial feeding method used for female *B. tabaci*. Figure made in Biorender©.

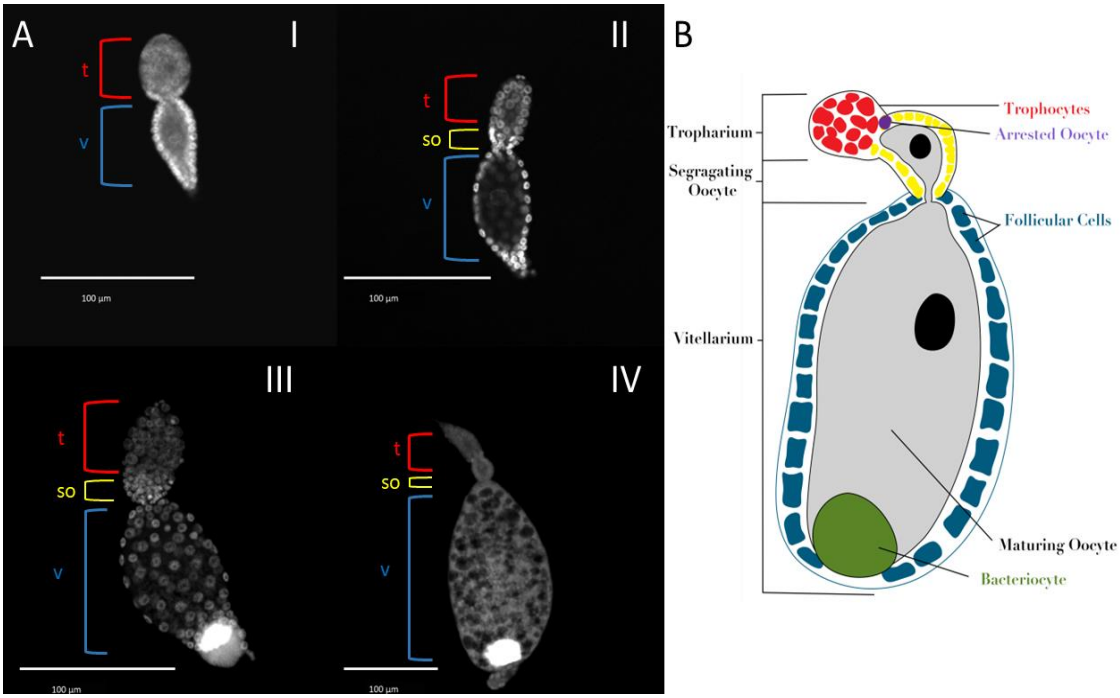

Figure 3.2. Diagrams of *B. tabaci* oocyte development. (A) Ovarioles with oocytes of different developmental stages as described by Guo et al. 2010. Brackets indicate regions of the ovariole (t = tropharium, so = segregating oocyte, v = vitellarium). (B) Labeled illustration of *B. tabaci* ovariole with mature oocyte. Brackets indicate regions of the ovariole. Colors represent cell types (red = trophocytes, purple = arrested oocytes, yellow = follicular cells surrounding the segregating oocyte, blue = follicular cells surrounding the mature oocyte, gray = oocyte, green = bacteriocyte). Black dots represent oocyte nuclei.

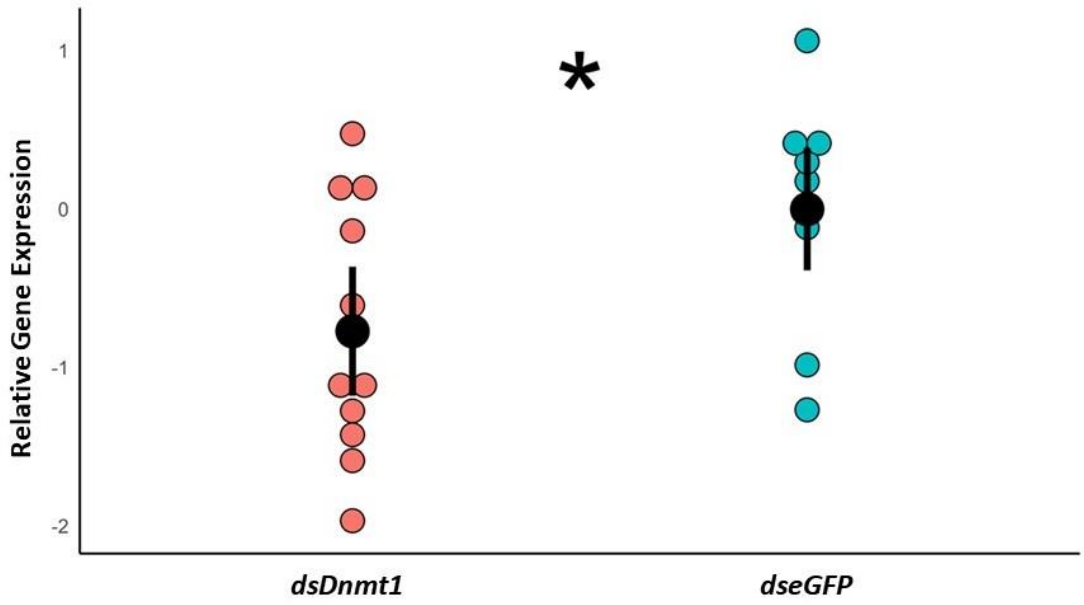

Figure 3.3. RNAi via feeding reduced *Dnmt1* gene expression. The values are represented as mean ±SE (black circle and bars, respectively) and as individual values (colored circles). Asterisks indicate statistical significance (* : $p \leq 0.05$).

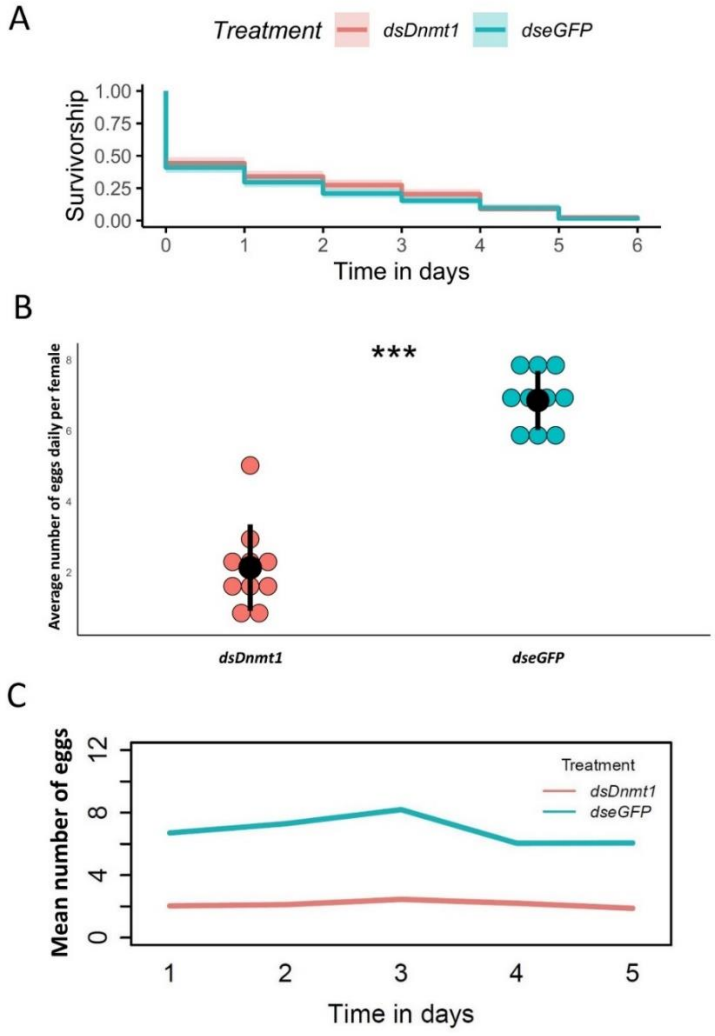

Figure 3.4. Reduced *Dnmt1* expression reduced fecundity, but not survivorship. (A) Survivorship for *dsDnmt1* and *dseGFP*-treated females was the same. (B) Fecundity was significantly reduced in the *dsDnmt1*-treated females. The values are represented as mean ±SE (black circle and bars, respectively) and as individual values (colored circles). Asterisks indicate statistical significance (*** : $p \leq 0.001$). (C) Fecundity did not change over time within treatment.

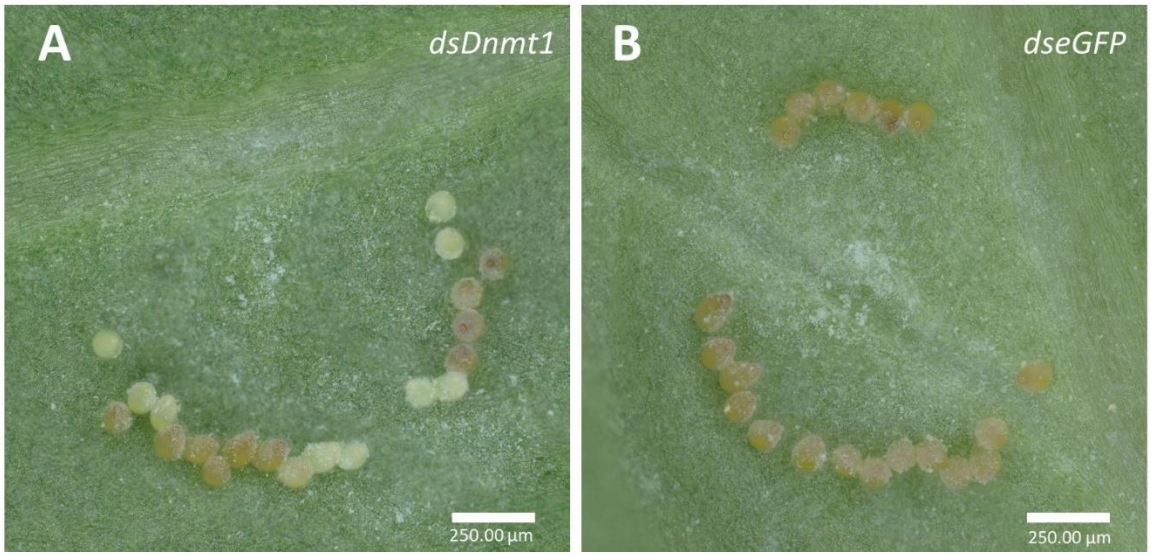

Figure 3.5. *Dnmt1* expression knockdown reduced egg viability. (A) Example egg clutch from *dsDnmt1*-treated female with viable (brown) and inviable (yellow) eggs at 5 days post-oviposition. (B) Example egg clutch from *dseGFP*-treated female with all fertile (brown) eggs at 5 days post-oviposition.

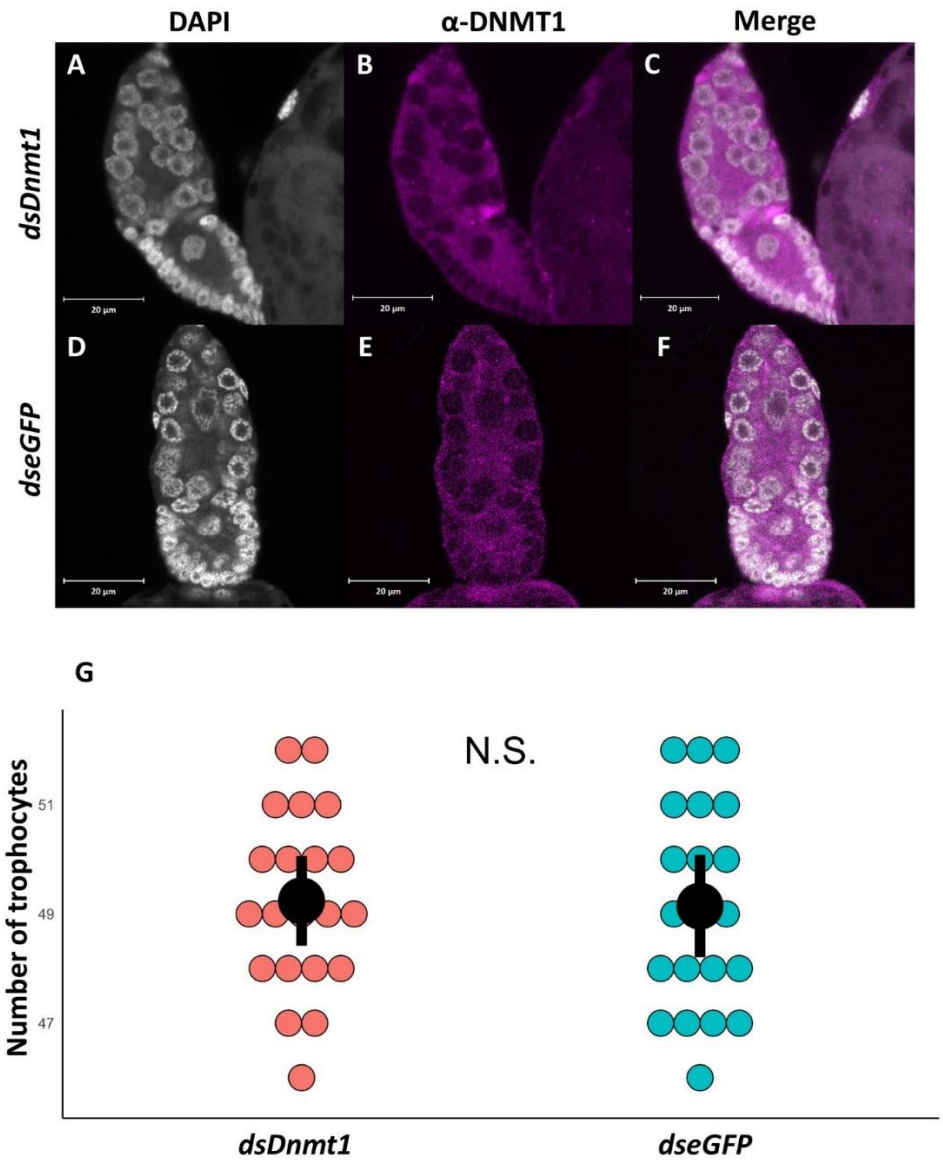

Figure 3.6. Reduction of *Dnmt1* did not impact the tropharium (trophocytes + segregating oocytes). (A-C) Tropharium from *dsDnmt1*-treated female stained with DAPI (A), CY5-labeled α-DNMT1 (B), and both (C). (D-F) Tropharium from *dseGFP*-treated female stained with DAPI (D), CY5-labeled α-DNMT1 (E), and both (F). (G) Number of trophocytes was not significantly different between treatments. The values are represented as mean ±SE (black circle and bars, respectively) and as individual values (colored circles). Asterisks indicate statistical significance (N.S.: $p \geq 0.05$).

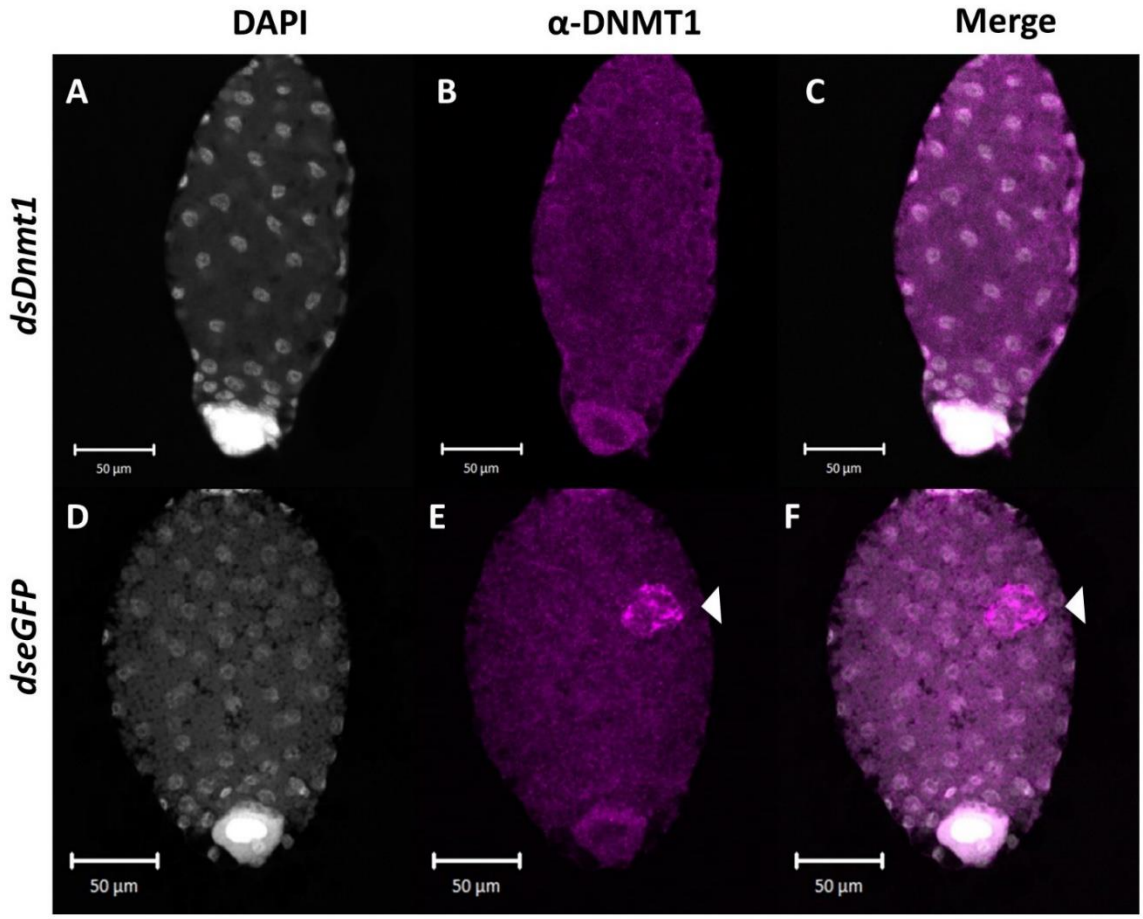

Figure 3.7. Reduction of *Dnmt1* prevented localization of DNMT1 into the maturing oocyte nucleus (A-C) Ovariole with maturing oocyte from *dsDnmt1*- treated female stained with DAPI (A), CY5-labeled α-DNMT1 (B), and both (C). (D-F) Ovariole with maturing oocyte with nucleus (indicated by white arrowhead) from *dseGFP*-treated female stained with DAPI (D), CY5-labeled α-DNMT1 (E), and both (F).

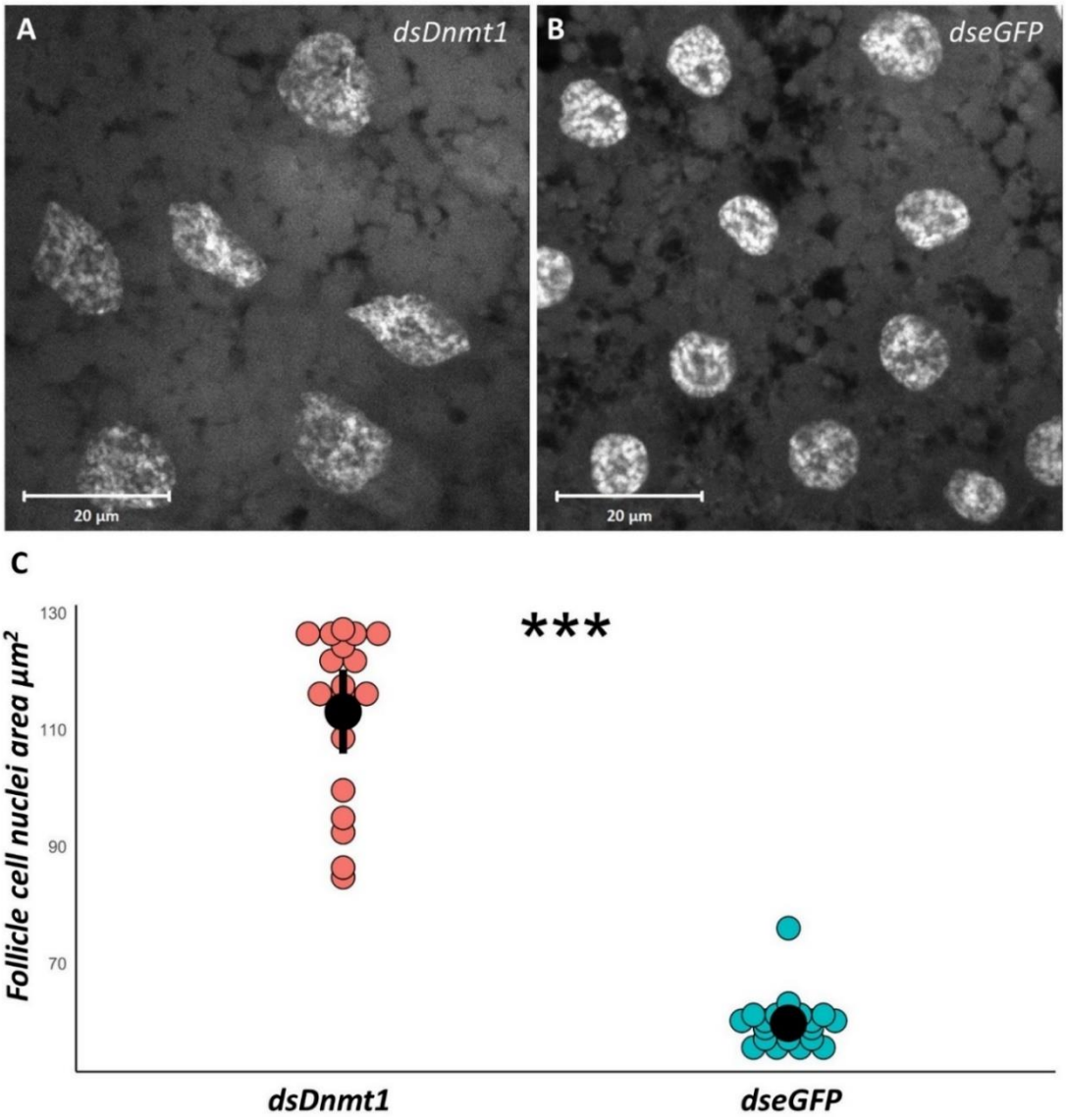

Figure 3.8. Reduction of *Dnmt1* changed the size of the follicular cell nuclei. (A) Follicular cell nuclei from *dsDnmt1*-treated female stained with DAPI. (B) Follicular cell nuclei from *dseGFP*-treated female stained with DAPI. (C) Follicular cell nuclei from *dsDnmt1*-treated females were significantly larger than those from *dseGFP*-treated females. The values are represented as mean ±SE (black circle and bars, respectively) and as individual values (colored circles). Asterisks indicate significance level (*** : $p \leq 0.001$).

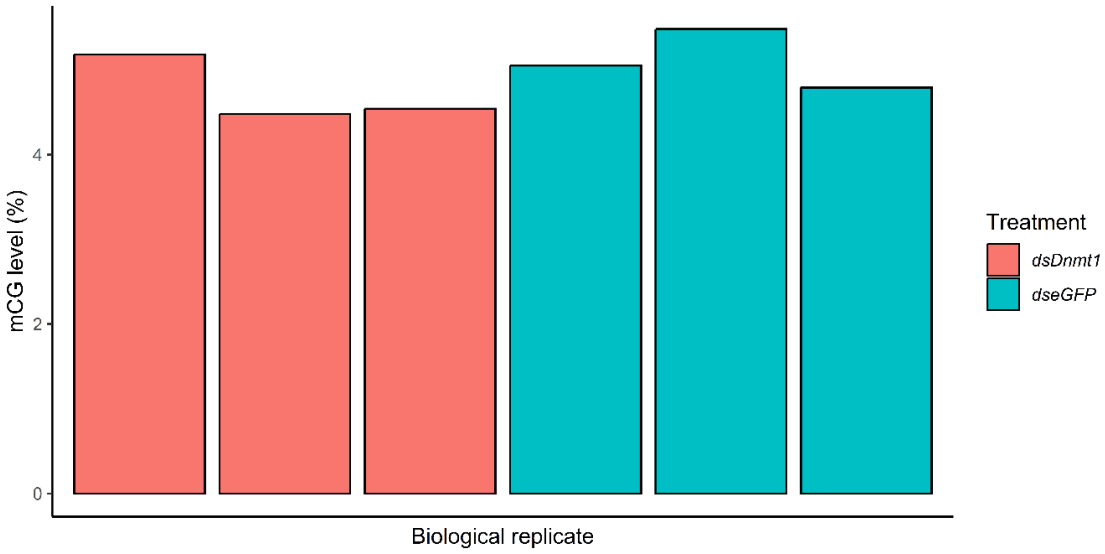

Figure 3.9. Reduction of *Dnmt1* did not reduce DNA methylation levels.

Each bar represents a biological replicate.

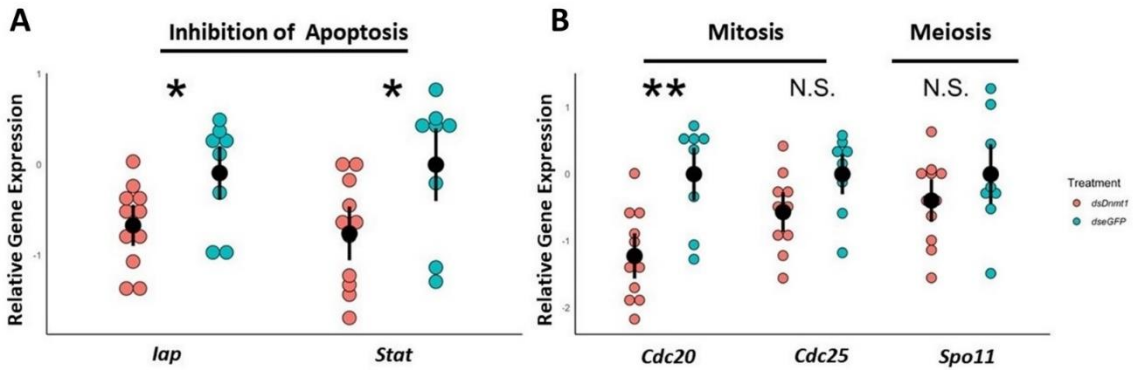

Figure 3.10. Reduction of *Dnmt1* affects expression of some cell maintenance genes. (A) Relative gene expression of apoptosis-related genes is significantly reduced following *dsDnmt1* treatment. (B) Relative gene expression of *Cdc20,* a mitosis gene, is significantly reduced following *dsDnmt1* treatment. Relative gene expression of *Cdc25* and *Spo11* is not significantly reduced. The values are represented as mean ±SE (black circle and bars, respectively) and as individual values (colored circles). Asterisks indicate statistical significance (N.S. : $p \geq 0.05$; * : $p \leq 0.05$; ** : $p \leq 0.01$).

CHAPTER 4

DNMT1 IS REQUIRED FOR EARLY EMBRYO DEVELOPMENT IN THE HAPLODIPLOID *BEMISIA TABACI* (HEMIPTERA:ALEYRODIDAE)[3]

[3]Shelby EA, McKinney EC, Cunningham, CB, Simmons AM, Moore AJ, Moore PJ. To be submitted to a peer-reviewed journal.

4.1 Abstract

The sweetpotato whitefly, *Bemisia tabaci* Gennadius, is a major economic pest that is difficult to manage with current strategies. Despite their importance and the available resources, little is known about factors that affect primary sex ratio and embryological development in this haplodiploid system. In this study, we show that DNA methyltransferase 1 (DNMT) is required for early embryogenesis in the *B. tabaci*. First, we show that loss of DNMT1 function does not result in differential embryo mortality or affect the adult sex ratio. We also identify key developmental stages during embryogenesis, which were shown to be consistent in both sexes. In addition, embryos produced from *dsDnmt1*-treated females failed to form a germ rudiment and had smaller-sized nuclei, suggesting inhibition of the cell cycle early in development. Though DNMT1's mechanism (methylation dependent or independent) remains elusive, our study provides insights into DNMT1's function based on *when* and *how* DNMT1 is needed, which provides insight for further development of management techniques.

Keywords: germ rudiment; reproduction; RNA interference; sex ratio; sweetpotato whitefly

4.2 Introduction

The sweetpotato whitefly, *Bemisia tabaci* Gennadius, is a globally important pest that causes billions of US dollars in damage to crops each year (Stansly & Naranjo, 2010; Inoue-Nagata et al., 2016; Czosnek et al., 2017). *Bemisia tabaci* is difficult to control as populations have developed resistance to most classes of insecticides (Horowitz et al.,

2020). The current common method used for *B. tabaci* pest management is the application of chemical insecticides like neonicotinoids (Horowitz et al., 2020), which negatively affect beneficial insect populations such as pollinators or *B. tabaci* natural enemies (Simmons & Jackson, 2000; Simmons & Abd-Rabou, 2005; Simmons & Shaabam, 2011). As such, considerable work has been directed towards developing new sustainable pest management strategies that exploit facets of whitefly biology (Shelby et al., 2020; Suhag et al., 2020). The economic importance of *B. tabaci* has led to an increase in the number of available technologies to make whitefly molecular studies more tractable such as sequenced genomes (Chen et al., 2016; Xie et al., 2017; Chen et al., 2019; Hussain et al., 2019), characterized methylomes (Cunningham et al., 2024), RNA sequencing data sets (Shen et al, 2023; Cunningham et al., 2024), transcriptomic and proteomic data sets (Yang et al., 2013), protocols for CRISPR (Heu et al., 2020) and RNA interference (RNAi; Dai et al., 2017; Gong et al., 2022; Jain et al., 2022; Shelby et al., 2023), life history summaries (Byrne et al., 1991; Aregbesola et al., 2020), and imaging methods (Luan et al., 2018, Bondy & Hunter 2019a; Shelby et al., 2023). Despite the availability of resources, aspects of whitefly biology, especially those that play a role in its success as a pest, continue to be understudied.

The main goal of pest management is to maintain the density of the pest population below the economic threshold. An important component of population growth is sex ratio (Bondy & Hunter 2019b). *Bemisia tabaci*, with its haplodiploid sex determination system, offers a unique opportunity to investigate factors that influence primary sex ratios and differential mortality of the sexes during embryonic development. In haplodiploid systems, virgin mothers only produce male offspring (haploid), whereas mated females produce

both males (haploid) and females (diploid). Sex of offspring from a mated female is determined by fertilization. While most *B. tabaci* population dynamic studies report on the factors that influence adult sex ratios (Pascal & Callejas, 2004; Cui et al., 2008; Crowder et al., 2010; Wang et al., 2012; Tsueda & Tsuchida, 2011; Parrella et al., 2013; Lü et al., 2014; Sun et al., 2014; Cass et al., 2016; Xiao et al., 2016) little is known about factors that influence primary sex ratios. Similarly, most studies that report "embryonic lethal" phenotypes following pesticide application do not include the developmental mechanisms that are being disrupted (Ishaaya, Mendelson, & Melamed-Madjar, 1988; Ishaaya & Horowitz, 1992), let alone if they are causing differential mortality of the sexes during embryonic development (Bondy & Hunter, 2019b). *Bemisia tabaci* also presents a challenge; unlike some pests, such as mosquitos, in which only females cause economic damage, both male and female *B. tabaci* need to be controlled because both sexes cause direct damage, and both cause economic damage through virus transmission during feeding (Morin, Atkinson, & Walling 2024). Therefore, effective pest management strategies need to reduce population levels and target both sexes.

A fruitful avenue for developing a successful whitefly management program is understanding *DNA methyltransferase I (Dnmt1)* function. Although the importance of DNA methylation in insects is unclear (Bewick et al., 2017; Duncan et al., 2022), DNMT1, the enzyme associated with maintaining DNA methylation, is vital for proper gametogenesis and embryogenesis (Zwier et al., 2011; Kay et al., 2018; Schulz et al., 2018; Bewick et al., 2019; Amukamara et al., 2020; Ventós-Alfonso et al., 2020; Washington et al., 2021; Arsala et al., 2022; Ivasyk et al., 2023; Shelby et al., 2023; Bidari et al., 2024). In *B. tabaci,* knockdown of *Dnmt1* results in the production of fewer eggs as well as fewer

eggs successfully hatching (Shelby et al., 2023). Although there was evidence that these eggs were abnormal before oviposition (Shelby et al., 2023), it is unclear whether the eggs failed to develop due to abnormalities during oogenesis that prevented the initiation of embryogenesis or if embryonic development began to occur but was prohibited from successfully progressing. Also, because previous experiments used exclusively virgin females, meaning that all embryos were male, it is unclear if loss of DNMT1 function results in differential mortality of the sexes during embryonic development.

The aim of our study was to determine the role of DNMT1 on embryogenesis in *B. tabaci*. First, we performed maternal RNAi followed by mating experiments and measured fecundity, viability, and sex ratios to determine if DNMT1 influences egg viability differences in mated or unmated females, and the sex ratio of offspring from mated females. Then we investigated the phenotypes associated with *dsDnmt1* treatment embryonic development using fluorescent microscopy, such as changes in nuclei morphology. As part of those experiments, we developed a timeline of key events in *B. tabaci* embryogenesis to identify if differences in sex affected timing of embryonic development as well as the effect of *Dnmt1* knockdown. We see no major differences in eggs produced from virgin or mated females and identify the specific developmental stage where *Dnmt1* appears to be required. For pest management strategies, our results suggest targeting gametes and embryos using *dsDnmt1* treatment are both viable targets from both virgin and mated females

4.3 Materials and Methods

Insect rearing

All individuals used in the experiments were taken from laboratory reared MEAM1 *B. tabaci* colonies. The colonies were started from wild caught populations collected from cotton fields in Tift County, Georgia, USA in 2018 (McKenzie et al., 2020). In the laboratory, we reared the colonies on collard plants (*Brassica oleracea*). We kept both *B. tabaci* and collards in an incubator at 27ºC with a 14:10 hour light: dark photoperiod and constant temperature and photoperiod throughout experiments. We used virgin females 3-5 days post adult eclosion for all experiments. To ensure the correct age and mating status, we removed newly-eclosed females from nymph colonies within 24 hours of adult eclosion. For experiments that required mated females, we paired virgin females 3-5 days post adult eclosion with males 3-5 days post adult eclosion.

Maternal RNA interference (RNAi)

We prepared double stranded interfering RNA and performed the knockdown protocol according to Shelby et al. (2023). Briefly, we made DNA templates of *Dnmt1* (our RNAi knockdown treatment) and *eGFP* (our RNAi control treatment) using PCR amplification with gene-specific primers and 500 ng/µl RNA (Table 1). We synthesized sense and antisense RNA in a single reaction using the Ambion MEGAscript RNAi kit (ThermoFischer Sci, Waltham, MA, USA) following the manufacturer's protocol. Following extraction and ethanol precipitation, we aliquoted the double-stranded RNA (dsRNA) and stored it at -80ºC until use.

We treated virgin females 3-5 days post adult eclosion with a dsRNA solution using an artificial feeding mechanism previously described in Shelby et al. (2023). The dsRNA

feeding solution contained green food coloring (McCormick & Company, Baltimore, MD, USA). The food coloring allowed us to confirm if a female had fed on the solution. If the females did feed on the solution, their abdomens would appear green when observed under a microscope. We only used females in these experiments which we confirmed had imbibed the dsRNA solution.

Egg collection, development, and adult sex ratios

Following feeding, we placed dsRNA-fed females on 15 cm tall collard plants. As an RNAi control group, we placed untreated (not fed dsRNA) females 3-5 days post adult eclosion on 15 cm tall collard plants. Each plant harbored 50-100 females. For the experiments with mated females, the females were placed on 15cm tall collard plants with an equal number of males. We checked plants for eggs every six hours for five days. If eggs were present, we removed adults from that plant and placed the adults on a new plant. We checked plants for eggs every six hours .We recorded the number of eggs from mated untreated, *dsDnmt1*-treated, and *dseGFP*-treated females daily.

For fecundity assays, viability assay, and adult sex ratio assays, all with mated females, we allowed eggs to develop into adults on the collard plants. Similar to what is described in Shelby et al. (2023), we recorded the number of eggs deposited, the number of eggs that displayed developmental progress (chorion pigmentation), and the number of eggs that hatched. We maintained individuals on the same plant from hatching until adult eclosion. After adults emergence, we removed all adults from plants and recorded the numbers of males and females.

To establish key developmental timelines during embryogenesis, we sampled eggs from virgin and mated untreated females at approximately 6-, 12-, 18-, 24-, 48-, 72-, and 84- hours post oviposition (PO). We defined the developmental stages based on descriptions of embryogenesis in *Oncopeltus fasciatus* (Dallas) (Panfilio et al., 2006; Chipman, 2017) and *Acyrthosiphon pisum.* (Harris) (Miura et al., 2003). To identify the effects of *Dnmt1* knockdown on embryogenesis, we collected eggs from mated and virgin females that were either treated with either *dsDnmt1* or *dseGFP*. For knockdown assays, we used eggs at approximately 6-, 18-, and 24- hours PO as these were considered critical times for development. We removed collard leaves with whitefly eggs and either immediately removed the eggs from the leaves or kept the eggs on the leaves until they were the appropriate age. We wrapped the leaves in a damp paper towel and placed them in a sealed petri dish in an incubator at 27ºC to prevent desiccation.

Image acquisition and analysis

We removed eggs from collard leaves using a probe mounted with a minute pin. After removal from the leaves, we dechorionated eggs by soaking them in 5% sodium hypochlorite for 15 minutes. Following the removal of the chorion, we washed embryos in PBS and incubated in 32% paraformaldehyde overnight at 2°C. To visualize nucleic acids, we used 1 μL 0.5 μg/mL, DAPI in PBS (Thermofisher Scientific, Waltham, MA, USA). We mounted the stained embryos with Mowiol 4-88 mounting medium (Sigma-Aldrich, St. Louis, MO, USA). We imaged the embryos with a Zeiss LSM 880 Confocal Microscope (Zeiss). Z-stack maximum projection images were taken. Only global image adjustment

features (such as brightness and contrast) were used. All confocal images were falsely colored.

We successfully processed and analyzed images from a total of N = 149 embryos, which included N = 100 embryos from untreated mated females, N = 14 embryos from virgin *dseGFP*-fed females, N = 10 embryos from virgin *dsDnmt1*-fed females, N = 15 embryos from mated *dseGFP*-fed females, and N = 10 embryos from mated *dsDnmt1*-fed mated females. For nuclei measurements, we used the ImageJ measuring software (Version 1.54i by FIJI). For embryos with less than 30 nuclei, we measured all nuclei. For embryos with more than 30 nuclei, we measured 30 randomly-selected nuclei.

Statistical analyses

We conducted a two-way ANOVA, followed by a Tukey HSD Post-Hoc test to compare the effect of treatment and mating status on the number of eggs produced. We conducted a repeated G-test to compare the effect of treatment and mating status on egg viability. We also conducted a repeated G test to compare the effect of treatment on adult sex ratio. For both replicated G-tests, we first performed a G-test of independence to give a heterogeneity G- value. Because the heterogeneity G-values were not significant (there were no difference across trials), we pooled data for final G test analyses. We performed a G test-Goodness of Fit Test to determine whether the proportion of embryos at a given developmental stage was equal between virgin and mated females. We used a two-way ANOVA to compare the effect of treatment and mating status on nuclei size. For data analysis, we used Base R in RStudio (RStudio Team 2023). We used the ggplot package for data visualization (Wickman 2016).

4.4 Results

Dnmt1 knockdown reduces fecundity and viability in virgin and mated females

DsRNA treatment had a significant effect on the number of eggs laid (ANOVA; $F = 13.815$, df = 2,24, $p < 0.0001$; Fig. 1A; Table 2). Specifically, *dsDnmt1*-treated females laid fewer eggs than untreated (TukeyHSD; p = 0.0002) and *dseGFP*-treated (TukeyHSD; p = 0.0005) females. There was no significant difference in the number of eggs laid between untreated and *dseGFP*-treated females (p = 0.932; Fig 1A). Mating status did not have a significant effect on the number of eggs produced ($F = 0.547$, df = 1,24, p = 0.467; Table 2). There was no significant interaction between treatment and mating status on the number of eggs laid ($F = 0.223$, df = 2, 24, p = 0.802; Table 2).

The *dsDnmt1* treatment significantly influenced egg viability in both virgin (G-test; G = 108.525, df = 2, P < 0.0001) and mated females (G = 72.739, df = 2, P < 0.0001) females (Fig. 1B). Overall, egg viability was reduced by about a third in *dsDnmt*-treated females compared to untreated and *dseGFP*-treated females (virgin: G = 5.066, df = 4, P = 0.281; mated: G = 5.143, df = 4, P = 0.273), but this heterogeneity was driven by one or two replicates in both virgin and mated untreated females, whereas the variation was more extreme in the *dsDnmt1*-treated virgin and mated females (Fig. 1B).

Dnmt1 knockdown does not affect adult sex ratio of offspring

In contrast to egg viability, there was no statistically significant effect of treatment on sex ratio of offspring produced by mated females (G = 1.970, df = 2, P = 0.373; Table 3) and thus no evidence of differential effects of treatment on viability to adulthood. Again,

there was significant heterogeneity among the untreated replicates (G = 23.532, df = 4, P < 0.0001) and the *dseGFP* replicates (G = 30.453, df = 4, P < 0.0001) but not among the *dsDnmt1* replicates (G = 3.657, df = 4, P = 0.454). This variation is likely to be random, given the differences were not unidirectional. In all the treatments, 100% of the offspring produced by virgin females were male.

Overview of development in embryos from untreated females

Like other hemipterans, the *B. tabaci* embryos developed as a syncytium during the cleavage stage from 0-12 hours post-oviposition (PO). Syncytial cleavage began initially at the center of the egg around (Fig. 4.2A) and expanded outward towards the periphery (Fig. 4.2B). During this time the nuclei divided synchronously, and the size of the nuclei varied as they rapidly progressed through the cell cycle (as seen in Fig. 4.2A-C). After reaching the periphery, the nuclei were regularly-spaced and approximately the same size, suggesting that they were undifferentiated (Fig. 4.2D). During the blastoderm stage (12-18 hours PO), two cell populations began to segregate: blastoderm cells and extraembryonic cells. The blastoderm cells concentrated towards the posterior region near the bacteriocyte and began to form the germ rudiment (Fig. 4.2E).Gastrulation took place from 18 - 48 hours PO. This stage began with the condensation of the germ rudiment and invagination towards the center (Fig. 4.2F). The bacteriocyte also moved towards the center. As a result of this movement, the embryo extended with the cephalic region at the posterior region of the egg. Segmentation occurred approximately 48-84 hours PO. Cephalic and thoracic appendages were visible first (Fig. 4.2G), followed by abdominal segments which housed the bacteriocyte. For most of segmentation, the embryo was immersed in yolk. Subsequent

stages of growth and development occurred until approximately 120 hours PO when the embryo hatched as a nymph. These stages included elongation of appendages and the dorsal closure of the embryo (Fig. 4.2H). During this stage, visualization with fluorescent nuclear staining was disturbed, likely due to secretion of the cuticle. There was no difference in developmental timing based on mating status as the proportion of embryos at a given developmental stage was equal between virgin and mated females (G-Test: G = 0.442, df = 9, P = 1).

Dnmt1 knockdown affects early embryogenesis

Maternal RNAi knockdown of *Dnmt1* produced lethal phenotypes in 70% of embryos. These embryos did not develop beyond the blastoderm stage (18 hours PO) and failed to form a germ rudiment (Fig. 4.3). The remaining 30% of embryos did not have a knockdown phenotype. At 24 hours PO, embryos from virgin and mated *dsDnmt1*-treated females had similar knockdown phenotypes (Fig. 4.3C & 4.3I). In contrast, embryos from virgin and mated *dseGFP*-treated females proceeded to undergo gastrulation at 24 hours PO (Fig. 4.3F & 4.3L). These results suggest that *Dnmt1* may be required for germ rudiment formation.

Both female mating status and treatment affected nuclei size during pre-blastoderm development (Fig. 4). Initially, female mating status had an effect on nuclei size with embryos from mated *dseGFP*-treated females being significantly larger than embryos from virgin *dseGFP*-treated females (F = 6.040, df = 1,774, p = 0.014). Knockdown of *Dnmt1* also significantly reduced nuclei size (F = 162.384, df = 1,774, p < 0.001). Additionally, there was a significant interaction between female mating status and knockdown of *Dnmt1*

(F = 10.436, df = 1,774, p = 0.001) with the amplitude of the change in size is larger in nuclei of embryos from virgin females due to their larger size in *dseGFP* embryos.

4.5 Discussion

In this study, we show that DNA methyltransferase 1 (DNMT) is required for early embryogenesis in the *B. tabaci*. First, we show that loss of DNMT1 function does not result in differential embryo mortality or affect the adult sex ratio. Because virgin females only produce male offspring and mated females produce offspring of both sexes, comparing fecundity and offspring mortality between virgin and mated females can determine if differential mortality based on sex is occurring (Bondy & Hunter, 2019b). Our study demonstrated that the effects of *Dnmt1* was not sex specific as both virgin and mated *dsDnmt1*-treated females laid fewer eggs and had fewer viable eggs. The knockdown result is strengthened by the lack of difference between untreated and *dseGFP*-treated females between and within mating groups. These results suggest that the function of DNMT1 is not sex specific, and *dsDnmt1* treatment does not affect the sex ratio in *B. tabaci* populations.

We catalogued key events in *B. tabaci* embryonic development, for which there are no detailed embryological studies published. Based on our descriptions of the timing and morphogenesis during *B. tabaci* embryogenesis, our results indicate that the development of this species is comparable to other insect systems such as *Oncopeltus fasciatus* (Dallas) (Liu & Kaufman, 2004; Panfilio et al., 2006), *Rhodnius prolixus* Stål (Berni et al., 2014), *Gryllus bimaculatus* De Geer (Donoughe & Extavour, 2016), *Murgantia histrionica* (Hahn) (Hernandez, Pick, & Reding 2020), *Nilaparvata lugens* Stål (Fan et al., 2020). This

suggests the pattern of development may be conserved among hemipterans and perhaps hemimetabolous insects in general. Based on the timing of developmental stages in embryos from both virgin and mated females, our study indicates that sex does not affect developmental timing. This result aligns with what has been observed in the hymenopteran *Nasonia diterpenes* (Walker), another obligate haplodiploid system but one that is holometabolous (Arsala & Lynch, 2017).

Knockdown of *Dnmt1* prevented *B. tabaci* embryos from forming a germ rudiment. Cleavage may also have been affected as loss of *Dnmt1* resulted in smaller nuclei. In other insect taxa, loss of *Dnmt1* has resulted in similar embryo failure during the blastoderm phase or before gastrulation (Schulz et al., 2018; Ventós-Alfonso et al., 2020; Arsala et al., 2022). This suggests that *Dnmt1* plays an evolutionarily conserved role specifically in early embryogenesis in insects. Indeed, expression of *Dnmt1* peaks during early embryogenesis before gastrulation and decreases as development progresses (Arsala et al., 2022). Also, given that loss of *Dnmt1* affected embryos of both sexes before the blastoderm phase, it is likely that ploidy (and therefore amount of DNA) may not dictate DNMT1 function. This suggests that DNMT1 may function similar to a maternal factor and may be regulated by the timing of the maternal clock. Also, the timing and phenotype of the *Dnmt1* knockdown embryos could indicate that these embryos are not capable of completing either the transition from maternal to zygotic transcription or the mid-blastula transition, a stage in development characterized by changes in the cell cycle and loss of synchronous cell divisions (Vastenhouw et al., 2019). We indirectly observed cell cycle changes via the changes in nuclei size observed during knockdown. However, future studies are needed to

decipher how DNMT1 interacts with DNA and other components to understand its mode of action.

Based on the results of this study (and those in Shelby et al., 2023), we suggest that *Dnmt1* is an appropriate target gene for sustainable management of the haplodiploid pest *B. tabaci*. Loss of DNMT1 function through ingestion of *dsDnmt1* reduces the number of eggs laid as well as reduced the viability of eggs. In addition to reducing the population, *dsDnmt1* treatment may effectively help to maintain the population dynamics and prevent economic damage through feeding and viral transmission.

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Table 4.1. Primer sequences used for dsRNA synthesis.

Gene	Sense Primer	Anti-sense Primer
Dnmt1	TCAATGATCATGATGAAAGGCCGCA	TGTCAGTGCTGACATTCCACACGGA
eGFP	CGAATTCACTAGTGATTTTACTTG	GCGGGAATTCGATTTGACC

Table 4.2. ANOVA table for effect of treatment and mating status on number of eggs laid.

	SS	*df*	*MS*	*F*	*p*-value
Treatment	7338	2	3669	13.815	0.0001 ***
Mating status	145	1	145	0.547	0.467
Interaction	118	2	59	0.223	0.802
Residual variance	6374	24	266		
Total	13975	29			

Table 4.3. Sex ratio of adult *B. tabaci* adults in relation to treatment group.

Treatment	Male	Female	Total
Untreated	122	100	222
dsDnmt1	17	17	34
dseGFP	76	116	192
Total	255	193	488

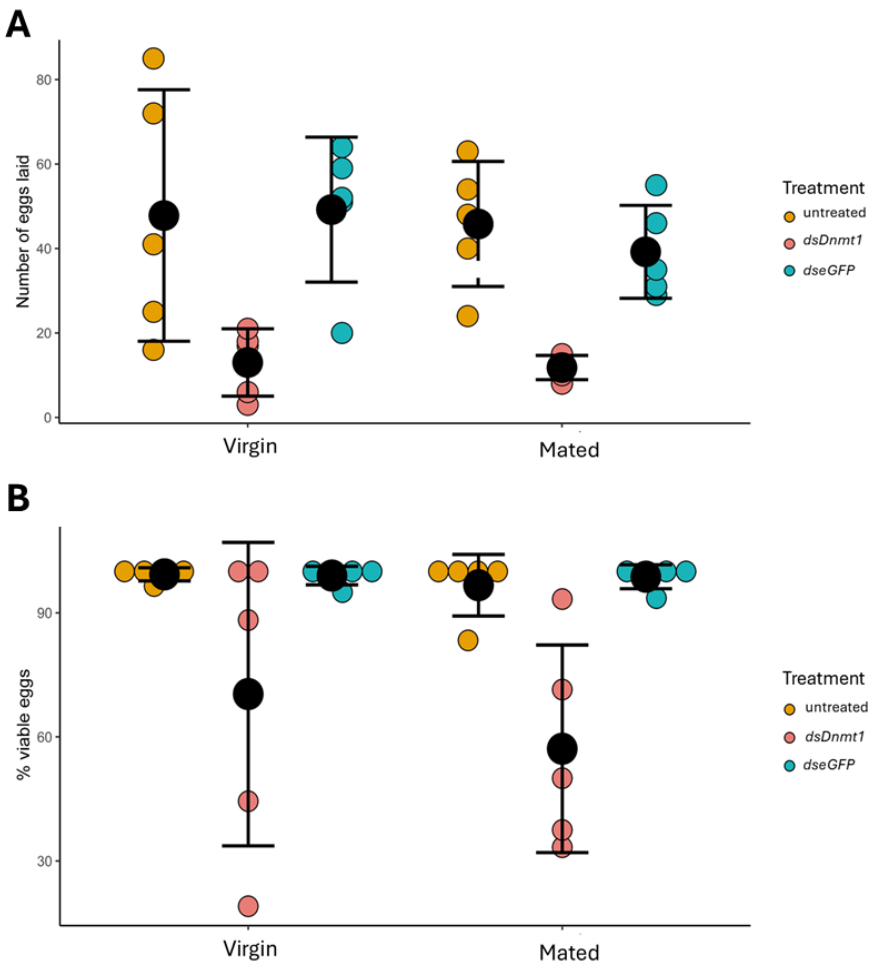

Figure 4.1. *Dnmt1* knockdown reduced fecundity and egg viability in virgin and mated *B. tabaci females*. (A) Fecundity was reduced in virgin and mated *dsDnmt1*-treated females. (B) Eggs laid by *dsDnmt1-treated* females had reduced viability. The values are represented as mean ± SE (black circle and bars, respectively) and as individual values (colored circles). For each treatment, N= 5 cages with 100 females per cage.

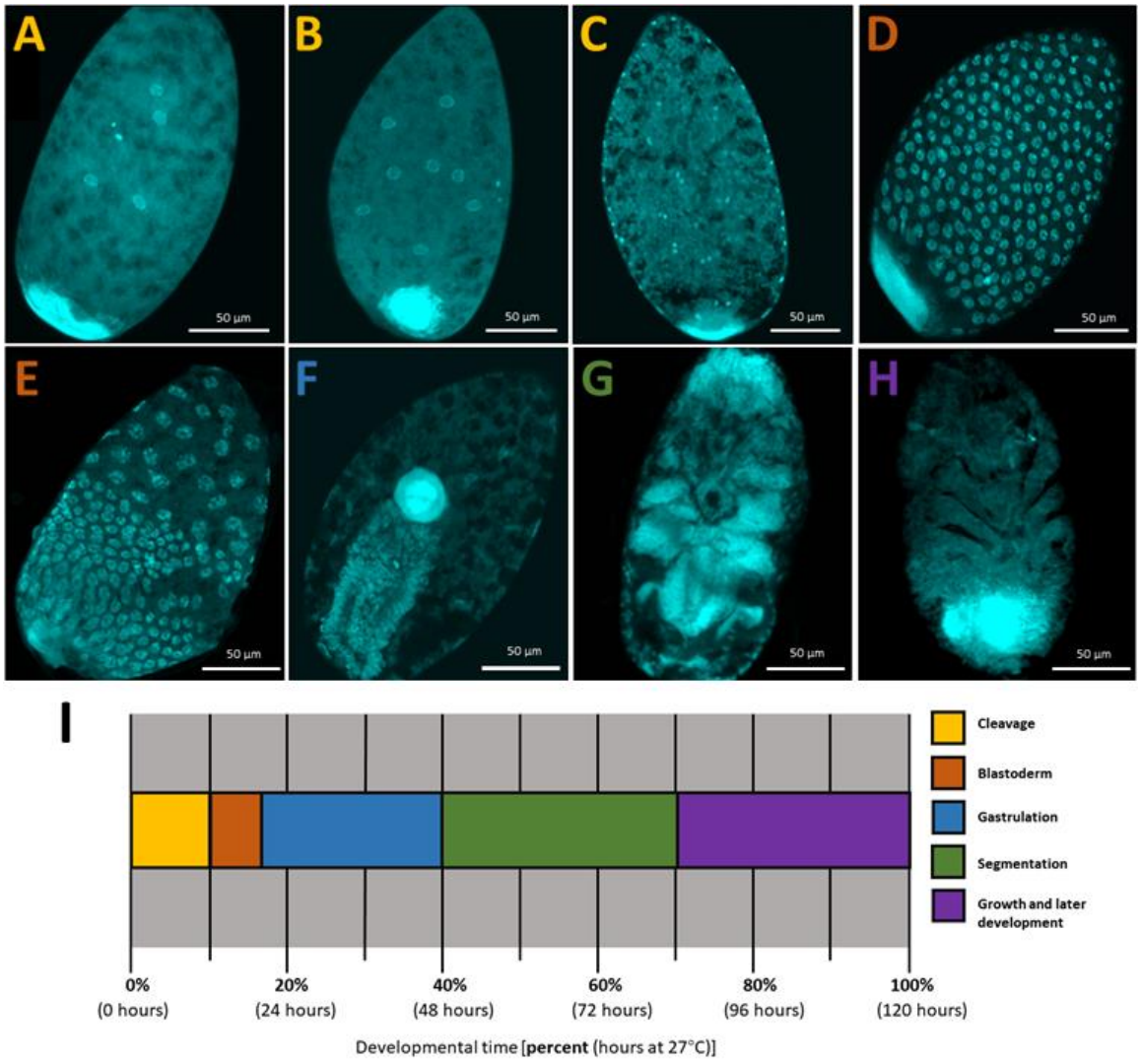

Figure 4.2. Illustration of key developmental stages during B. tabaci embryogenesis by DAPI nuclear staining: (A-C) cleavage, (D-E) blastoderm, (F) gastrulation, (G) segmentation, and (H) growth and later development. (I) A developmental timeline illustrating the onset and duration of stages examined in this study (color of letter denoting embryo image corresponds with the stage color on the timeline).

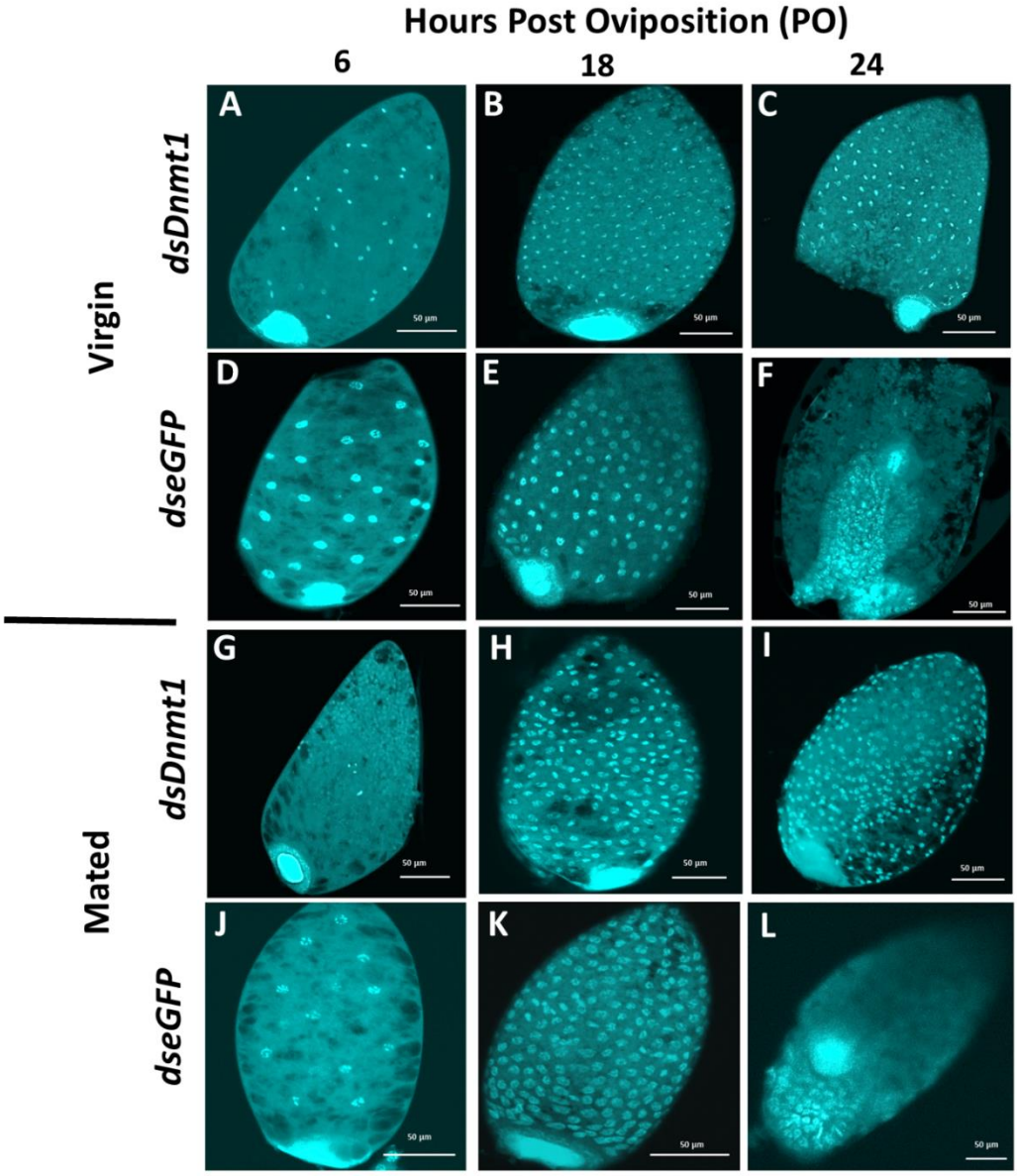

Figure 4.3. Knockdown of *Dnmt1* via maternal RNAi results in inability to form a germ rudiment at 24 hours post oviposition (PO). Images were taken at 6, 18, and 24 hours PO of embryos from virgin (A-F) and mated females (G-L) and *dsDnmt1*-treated females (A-C,G-I) and *dseGFP* -treated (D-F, J-L).

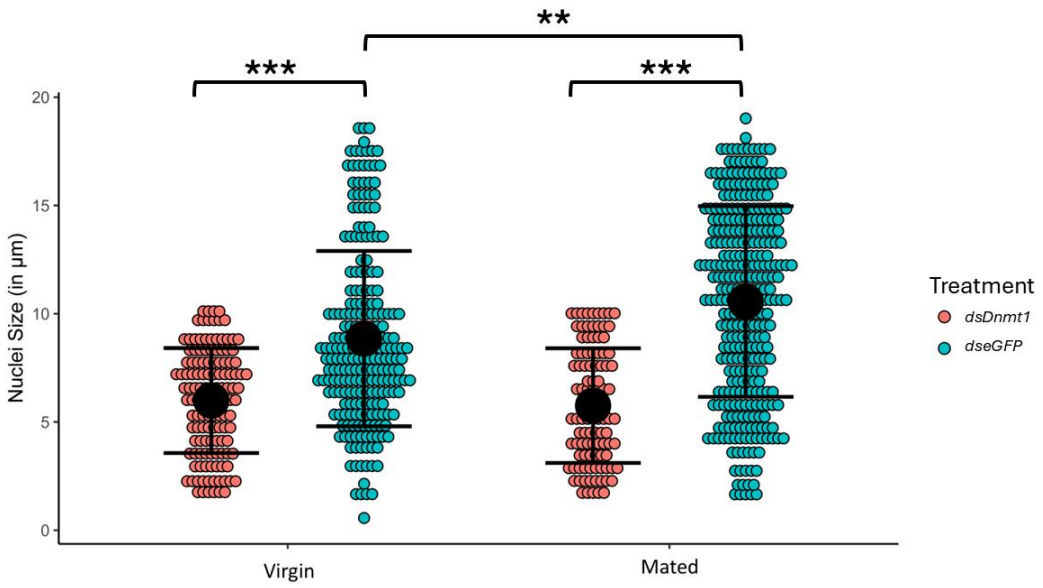

Figure 4.4. Loss of *Dnmt1* resulted in a smaller nuclei size in embryos from virgin and mated females. The values are represented as mean ± SE (black circle and bars, respectively) and as individual values (colored circles). Asterisks indicate statistical significance (**: $p \leq 0.01$; ***: $p \leq 0.001$).

CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

The function of DNMT1, the importance of DNA methylation, and the pathways that provide genome stability have been outstanding interconnected questions. The relationship between those components has been oversimplified into "DNMT1 methylates DNA, which regulates gene expression." But biology is rarely that elegant. Despite there being an almost universal role for DNMT1 in reproduction and development, the mechanism remains largely unknown because of the need to perfectly equate DNMT1 function with DNA methylation and gene expression in all organisms. In this dissertation, I have taken an integrative approach to identify *what* DNMT1 does in whiteflies and *when* DNMT1 is essential.

In Chapter 2, I suggest that *Dnmt1* could be used as a target gene for RNAi-mediated management of *B. tabaci* based on its conserved function in reproduction and embryogenesis. In Chapters 3 and 4, I demonstrate that loss of DNMT1 function *in B. tabaci* does indeed result in loss of fecundity and embryo viability. I also show that the phenotypes associated with this loss are consistent with what is observed in both invertebrates and vertebrates: loss of DNMT1 is associated with reduced and abberant gamete production and loss of DNMT1 results in embryo failure prior to or during gastrulation. Because there phenotypes were apparent despite DNA methylation levels being altered, my work suggests that DNMT1's importance during reproduction and

embryogenesis is likely unrelated to DNA methylation or regulating gene expression. Though it is not conclusive, based on the abberant nuclear phenotypes and expression of cell cycle genes, DNMT1 may be performing a more general function in genome stability, such as control of chromatin condensation.

In summary, this dissertation is a small step for DNMT1/DNA methylation research. Insights from this work provide another data point closer towards understanding the mechanism behind DNMT1's function using an organism that does not heavily rely on DNA methylation. The most important insight from the DNMT1/DNA methylation system in insects may be the expansion of the range of possibilities of mechanisms organisms use to ensure genome stability. In order to make advances towards identifying DNMT1's unknown mechanism, we as researchers need to accept when preconceived notions are incorrect, shed hypotheses, and turn to unconventional approaches. These approaches include continuing to investigate DNMT1 outside the context of DNA methylation, using direct measurements of DNA methylation instead of using loss of DNMT1 as a proxy, and visually investigating the embryonic phenotype.

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