THE ESSENTIAL ROLE OF DNMT1 IN GAMETOGENESIS IN THE LARGE MILKWEED BUG, *Oncopeltus fasciatus*

by

JOSHUA WASHINGTON

(Under the Direction of Patricia Moore)

ABSTRACT

Given the importance of DNA methylation in protection of the genome against transposable elements and transcriptional regulation in other taxonomic groups, the diversity in both levels and patterns of DNA methylation in the insects raises questions about its function and evolution. In this thesis, I show that the maintenance DNA methyltransferase, DNMT1, affects meiosis and is essential to fertility in male milkweed bugs, *Oncopeltus fasciatus*, while DNA methylation is not required in somatic cells. My results support the hypothesis that *Dnmt1* is required for the transition of germ cells to gametes in *O. fasciatus* and that this function is conserved in male and female gametogenesis. They further suggest that DNMT1 has a function independent of DNA methylation in germ cells. My results raise the question of how a gene so critical in fitness across multiple insect species can have diverged widely across the insect tree of life.

INDEX WORDS: DNA methylation, epigenetics, *dnmt1*, spermatogenesis, germ cells, *Oncopeltus fasciatus*

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JOSHUA WASHINGTON

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JOSHUA WASHINGTON

Major Professor:	Patricia Moore
Committee:	Kevin Vogel
	Mary Goll

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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DEDICATION

To myself. You did it Joshey Washie. #OneDegreeHotter

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Showcasing my work without giving the sincerest thank you to Dr. Patricia Moore would be wildly inappropriate. In short, I'm a much better scientist because of her and without her, my work would not have been accomplished.

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CHAPTER 1

INTRODUCTION

Investigations into the function of DNA methylation in *Eukarya* started in 1970 (Schmitz et. al 2019). After decades of research in vertebrate and plant systems, scientists widely accept DNA methylation to function in genome regulation and protection. During this time, one of the main enzymes responsible for methylating DNA was discovered: DNA methyltransferase I
(DNMT1) (Bestor et. al 1988). Through the methylation of transposable genetic elements and through transcriptional control, DNMT1 has been closely linked to genomic protection and regulation, respectively. However, in insects, the function of DNMT1 is not well known. *Drosophila melanogaster* does not have a methylated genome or DNMT1. Even in the entire dipteran order, DNA methylation nor the presence of DNMT1 has been detected. This, in combination with *D. melanogaster* being the canonical model for insect studies, led us to conclude insects as a whole do not have methylated genomes. However, recent evidence shows that the absence of DNMT1 in *Diptera* is not representative of every order within *Insecta*. In fact, the majority of insect orders have DNMT1 alongside methylated genomes (Bewick et. al 2017, Provataris et al. 2018). Recently, a loose association of DNMT1 with genome protection and regulation has been implicated in vertebrate systems (Kaslow et. al 1987, Goll and Bestor 2005), but interestingly enough, when looking at insects, the disconnect is even more drastic. Insect phylogeny highlights how DNMT1 and DNAmethylation do not have a perfectly proportional relationship. To add, DNMT1 was found to be necessary in an organism whose levels of DNA methylation are undetectable, thereby suggesting the enzyme functions independently of genome regulation and protection. Thus, the question arises: what exactly is the function of DNMT1 in insects?

Review of DNA Methylation

Across plants, animals, and fungi, regulation of gene expression and protection of the genome are accepted as the classic roles of DNA methylation. The specific bases targeted in DNA methylation are cytosine an adenine. However, such targeting is largely disproportionate as cytosine is the predominant base subject to methylation – particularly at its fifth position (Schmitz et. al 2019). Now known as 5-methylcytosine (5mC), *5mC* and *DNA Methylation* are used interchangeably since cytosine is the most common base in eukaryotic genomes to be chemically modified. However, in order for DNA methylation to occur, particular sequences must be present. CG is the main sequence leading to methylation, but taxa-specific sequences exist within *Eukarya* as well. As an example, CXX is exclusively found in plants (X = A, C, or T) (Bewick et. al 2016, Schmitz et. al 2019). Still, with any methylated sequence in *Eukarya*, it is associated with gene regulation and genome protection.

Monoallelic expression of imprinted genes and the inactivation of X chromosomes have been linked to DNA methylation in mammals. Imprinted genes were discovered in 1960 and more recently, studies using marsupials, rodents, and even humans have linked that phenomenon, alongside X-inactivation, to DNA methylation (Crouse 1960, Mohandas et. al 1981, Sado et. al 2000, Rens et. al 2010). Even in *Arabidopsis thaliana*, the canonical model of plant studies, the long-term inactivation of genes during cell differentiation and overall development is linked to DNA methylation (Adams et. al 2000, Jullien et. al 2006).

Regarding genome protection, DNA methylation has been tied to the silencing of transposable elements (TEs) (Schmitz et. al 2019). As mobile TEs are highly mutagenic and deleterious, methylating TEs in order to silence their activity is a plausible means of protection. In fungi, genome protection can also come in the form of mutation. Originally discovered in 1986, a system known as repeat-induced point mutation (RIP) was found in the filamentous fungus

Neurospora crassa (Selker et. al 2002). In action, RIP mutates repeat sequences and subsequently methylates them in order to maintain genome stability. Such a defense mechanism was later found in *Ascobolus immerses* and other fungi as well. Altogether, these studies successfully show a correlation between DNA methylation and the regulatory and protective functions of the genome.

Interestingly, there's evidence to suggest these functions are more loosely associated with DNA methylation than previously thought. More specifically, evidence suggests that marsupials have X chromosomes not regulated by DNA methylation (Kaslow et. al 1987). Their dosage compensation is accounted for by inactivation of the paternal X chromosome via methylation and in the opossum *Didelphis virginiana*, Kaslow et. al showed how expression of that X chromosome was still silenced despite its demethylation. This suggests mechanisms other than DNA methylation function in the inactivation of the X chromosome. Additionally, similar conclusions can be derived from studies with mice as their models. By using pMT(N)neo, a vector used to downregulate methylation enzymes in mouse embryos, Li et. al reduced their levels of DNA methylation. Although developmental delay occurred 8.5 days *post coitus*, Goll and Bestor assert how massive dysregulation is expected to lead to a more immediate effect (Li et. al 1992, Goll and Bestor 2005). In another study centered around mammalian development, the expression of tissue-specific genes was found not to be correlated with DNA methylation despite earlier reports claiming the opposite (Walsh, Bestor 1999). All things considered, transcriptional regulation and protection of the genome are roles of DNA methylation, but given the growing evidence, the functions of DNA methylation across *Eukarya* are not generalizable.

DNA Methyltransferases

DNA methyltransferases are the enzymes responsible for methylating DNA and they occupy two main classes: *de novo* and maintenance. *De novo* methyltransferases are enzymes responsible for establishing a methylated genome whereas maintenance methyltransferases conserve those already-established methylation patterns. In this light, *de novo* methyltransferases are proposed to be primarily active in early stages of development whereas maintenance methyltransferases would be active during cell division (Riggs 1975, Holiday and Pugh 1975). *De novo* methyltransferases fail to discriminate between hemi- and unmethylated DNA strands. Furthermore, these enzymes are most highly expressed in embryonic stem cells, thereby further supporting the need to establish DNA methylation patterns early on (Okano et. al 1999). When cells divide, those patterns need to be maintained. Unlike their *de novo* counterparts, maintenance methyltransferases do discriminate between hemi- and unmethylated strands of DNA . Maintenance methyltransferases will only methylate DNA if the preexisting condition of hemimethylation is present. Contextually, during semi-conservative replication of DNA, maintenance methyltransferases are recruited to the unmethylated daughter strand whose complementary strand is already methylated. Together, *de novo* and maintenance methyltransferases are the widely accepted factors responsible for methylating DNA.

Of the *de novo* and maintenance methyltransferases, some are shared among taxa while others are exclusive to a certain taxon. In plants, there are four types of DNA methyltransferases:

Methyltransferase 1 (MET 1), Chromomethylase (CMT), Domain Rearranged Methyltransferase 2 (DRM2), and DNA methyltransferase 3 (DNMT3) (Bewick et. al 2016, Yaari et. al 2019). MET1 one is a maintenance methyltransferase responsible for methylating cytosine in a CG sequence. Of the chromomethylases, there are two known types: Chromomethylase 2 (CMT2) and Chromomethylase 3 (CMT3). They are both maintenance methyltransferases but CMT2 and CMT3 recognize their own sequences: CXX and CXG, respectively. However, evidence is surfacing that suggests a *de novo* function of CMT3 as well (Wendte et. al 2019). Regarding *de novo* methylation in plants, DRM2 is responsible for establishing methylation in CXX sequences. As for DNMT3, no work has been done to understand its function or targeted sequences in plants. It's proposed that DNMT3 is understudied in plants due to its deficiency in angiosperms alongside its DRM2 homologue serving a *de novo* function (Yaari et. al 2019). In fungi, there are also four known DNA methyltransferases: DNA methyltransferase 1 (DNMT1), DNA methyltransferase 5 (DNMT5), DIM-2, and RID (Schmitz et. al 2019) Studies involving *N. crassa* show that fungal DNA methyltransferases don't recognize specific cytosine sequences but rather repeat cytosine sequences (Schmitz et. al 2019, Selker et. al 2002). More interestingly, there doesn't appear to be a mechanism of maintenance methylation – just *de novo* methylation at every round of cell division (Schmitz et. al 2019). In both vertebrates and non-vertebrates, only two main types of DNA methyltransferases exist: DNMT1 and DNMT3. DNMT3 establishes the methylation patterns while DNMT1 maintains them. However, they both recognize the same sequence: CG. Although the forms of DNA methyltransferases can be taxa-dependent, they all seem to perform one of two functions across *Eukarya*: establishing or preserving DNA methylation.

DNA Methyltransferases in *Hexapoda*

In examining the evolutionary history of DNA methyltransferases in hexapods, the overgeneralization of these enzymes serving regulatory and protective roles becomes quite clear. Generally, hexapods have two types of DNA methyltransferases: DNMT1 and DNMT3 (Schmitz et. al 2019). *Strepsiptera, Diptera,* and *Collembola* are exceptions because they lack the DNMT1 and DNMT3 and they also don't have methylated genomes (Bewick et. al 2017, Provataris et. al 2018). Although *Protura* has DNMT1, it lacks DNMT3 and a methylated genome as well (Provataris et. al 2018). Nevertheless, by considering just these orders of *Hexapoda,* we still cannot say a loose association exists between DNA methylation and its methyltransferases. However, when examining insects ranging from lower groups in "*Hemimetabola"* to some of the highest groups in *Holometabola*, we can. *Zygentoma, Odanata*, *Ephemeroptera, Lepidoptera*, and *Mecoptera* are disparate insect orders lacking DNMT3, but interestingly enough, they all have methylated genomes (Bewick et. al 2017, Provataris et. al 2018). Although this shows a clear disconnect between DNMT3 and the establishment of DNA methylation, we cannot say the same for DNMT1. The absence of DNMT3 can be explained by the possibility of DNMT1 functioning to both establish and maintain DNA methylation patterns. However, the validity of such an explanation is questioned when examining the DNA methyltransferase-DNA methylation relationship in *Microcoryphia, Dermaptera, Raphidioptera,* and *Neuroptera*. Although DNMT3 is absent and DNMT1 is present in these orders, none of them have a methylated genome, thereby failing to strongly support a dual *de novo*-maintenance function of DNMT1 (Provataris et. al 2018). The variations in methylation patterns coupled with their intermittent presence of associated DNA methyltransferases suggest DNMT1 and DNMT3 do not function to prevent genetic mutations, maintain genomic stability, or regulate gene expression in insects. The question then arises: what exactly is their function?

In an attempt to answer this, the role of DNA methylation in insects has been investigated. These studies suggest DNA methylation has diverse functions including contributions to foraging behavior, sociality, diapause, and more (Lockett et. al 2012, Sasibhushan et. al 2013, Bonasio et. al 2012, Cullen et. al 2017). With foraging in particular, whether or not a protein kinase geneis methylated differs amongst worker honey bees (Lockett et. al 2012). Foraging and nursing are two tasks completed by workers and the protein kinase gene in foragers is consistently more methylated than that same gene in nurses. Furthermore, DNA methylation has been associated with sociality in hymenopterans. Because DNA methylation patterns were found to be hive-specific, they were linked to the identity of a superorganism in honeybees (Lockett et. al 2012). In ants, analyzing the methylomes of *Camponotus floridanus* and *Harpegnathos saltator* led to the discovery of methylation differences among the sexual castes of ant colonies: active queens, virgin queens, and males (Bonasio et. al 2012). In a microarray analysis, DNA methyltransferases were found to be upregulated when silkworms were in diapause, thereby tying it to DNA methylation (Sasibhushan et. al 2013). Nevertheless, with all of these studies, their findings are merely correlational.

More recent work presents notable findings that contradict the aforementioned ones. Given optimal environmental conditions, females of *Bombyx mori* will lay eggs that enter diapause in the embryonic stage (Yamshita 1996). However, when exposed to 5-azacytidine, an inhibitor of DNA methylation, the proportion of embryos entering diapause did not change (Yuichi et. al

2016). Since there was no change in diapause activity, it appears to have a weak relationship with DNA methylation which, in turn, appears to also have a weak relationship with sociality. When accounting for solitary butterflies that have methylated genomes, subsocial flies that do not, and social hymenopterans that have no relationship with DNA methylation patterns, dissociating sociality from DNA methylation within insects as a whole seems appropriate (Bewick et. al 2017). If sociality were a function of DNA methylation, then its presence would expectedly relate to sociality in any of its forms: solitary, communal, subsocial, or eusocial. Through comparative tests, phylogenetic analyses show that it does not (Bewick et. al 2017). Though most of these studies are also correlational, their disagreement with previous studies highlights the importance of showing a causative effect, for the functions of DNA methyltransferases remain largely unknown.

DNMT1

Fortunately, recent functional analyses provide clearer insight into the function of DNMT1. Studies involving *Nilaparvata lugens* (brown planthoppers), *Nasonia vitripennis* (jewel wasps), and *Tribolium castaneum* (red flour beetles) prove DNMT1 is essential for reproduction (Zwier et. al 2012, Zhang et. al 2015, Schulz et. al 2018). In *N. lugens*, developmental expression profiles show gravid females displayed the highest expression of both DNMT1 and DNMT3 when compared to larvae, male adults, and virgin females (Zhang et. al 2015). After downregulating DNMT1 and DNMT3 using RNA interference (RNAi), a reduction in female fecundity resulted, thereby confirming these DNA methyltransferases were in fact responsible for either gametogenesis or embryogenesis. In other insect models, studies also using RNAi suggest DNMT1 is the sole DNA methyltransferase responsible for embryogenesis. In *N. vitripennis*, only a deficiency in DNMT1a (a paralog of DNMT1) led to embryonic lethality despite the downregulations of DNMT3 and DNMT1c (another DNMT1 paralog) (Zwier et. al 2012). Similarly, in *T. castaneum*, developmental arrests of the embryo resulted from the downregulation of DNMT1. As *T. castaneum* naturally lacks DNMT3, the only possible DNA methyltransferase responsible for an embryonic contribution be DNMT1. These studies successfully identified a function for DNMT1, but more interestingly, that function may be independent of DNA methylation. In *T. castaneum*, there have been no detectable levels of DNA methylation, suggesting it naturally lacks a methylated genome. Why then is an enzyme responsible for DNA methylation essential in an organism seemingly without it? Such a paradox suggests an alternative role of DNMT1.

Our work on *Oncopeltus fasciatus* (large milkweed bugs) parallels that claim. Embryogenesis, oogenesis, and follicle cells all center around reproduction: a process compromised when DNMT1 is downregulated via RNAi in adult females of *O. fasciatus* (Bewick et. al 2019). In the first 8 days of treatment, eggs laid by both controls and DNMT1-deficient females were quantitatively similar but qualitatively dissimilar. 93% of the eggs laid by controls initiated embryogenesis while only 4% of the eggs laid by DNMT1-deficient females did the same. Of that 4%, none completed embryonic development unlike their control counterparts. With respect to oogenesis, 10 days into treatment, controls continued laying eggs while DNMT1-deficient females stopped. When examining their ovarian follicles, the compromise in egg production was even more evident. In the vitellarium, controls had normally developing eggs while those of DNMT1-deficient females were replaced with a yolk-like substance. This, in combination with obstructed follicle cells, led us to conclude choriogenesis was defective. Due to the absence of DNMT1 and the subsequent demethylation of ovarian genomes

(Bewick et. al 2019), these reproductive phenotypes resulting from genome dysregulation is an understandable hypothesis. However, using RNA-seq, we showed gene expression was not impacted on the transcriptome level and there was little evidence of TE movement.
As a result, these reproductive phenotypes seemed to tie more to the absence of DNMT1 than to gene dysregulation or genome instability.

DNMT1 was downregulated in not only ovarian tissue, but somatic tissue as well – specifically that of the head, gut, and thorax (Bewick et. al 2019). However, DNA demethylation did not occur within the soma. In this light, a compromise specific to reproduction could have been possible only because ovarian tissue is the only cell type undergoing sufficient proliferation in adults to have demonstrable demethylation. Videlicet, cell division gives way to the passive loss of DNA methylation and its subsequent phenotype. Despite the downregulation of DNMT1 within the adult soma, a somatic phenotype will not manifest because somatic tissue proliferates at a considerably lower rate, if any, in adulthood. To address this issue, we treated females of *O. fasciatus* in their larval stages (Amukamara et. al 2019). Once they were adults, we assessed their development, longevity, and ovarian follicles. Regarding development, controls and DNMT1-deficient females had an equal chance of surviving to adulthood. From both treatments, 80% of female larvae emerged as adults. In addition, no observable morphologicaldefects were detected in DNMT1-deficient females. Upon adult emergence, their overall body plan was similar to that of control females: normal. With respect to longevity, females of both treatments showed no difference in their lifespans. We'd expect an impact on development and longevity in females whose somatic genomes were demethylated, but no such impact was observed. Conclusively, DNMT1 had a germline-specific function, but we examined the ovarian follicles to identify a more specific impact on reproduction. Compared to the oogenic compromise reported by Bewick et. al 2019, we saw an exacerbated defect in oogenesis when downregulating DNMT1 in female larvae. More specifically, oocytes were not even produced. Notably, there was one DNMT1-deficient female that laid eggs, but she laid 86 compared to controls laying 200 on average. When looking at images from microscopy, trophocytes and pedicels appeared normal in DNMT1-deficient females while their germ cells were disrupted and dying. Not only did developmental, lifespan, and ovarian data support a germline-specific function of DNMT1, but like *T. castaneum*, the relationship, if any, between DNMT1 and DNA methylation was found to be loose.

DNMT1 in Males of *Oncopeltus fasciatus*

When Amukamara et. al used RNAi to downregulate DNMT1 in females of *O. fasciatus*, their germ cell development was compromised (Amukamara et. al 2019). Since both males and females of *O.fasciatus* undergo gametogenesis, I investigated whether or not DNMT1 has a similar effect in spermatogenesis for my thesis research.

Also leading to this investigation was the paucity of research involving DNMT1 in male insects. Few functional analyses have been conducted on males, for the ones that do exist suggest DNMT1 serves no function in males at all. When Schulz et. al downregulated DNMT1 in males of *T. castaneum*, no effect on male fertility was observed (Schulz et. al 2018). However, when considering these beetles were treated during their pupal stage, the experimental design seems not to account for the sexual development of their model. As *Holometabola* already have spermatocytes in their larval stages, the onset of meiosis may begin before the pupal stage

(Economopoulos and Gordon 1971, Lago et. al 2020). After going through larval development, completing prepupal stages, and entering the pupal stage, it could be possible that *T. castaneum* already has a considerable supply of mature sperm once they are pupae. Therefore, a false negative result could have been observed when Schulz et. al downregulated DNMT1 in a stage where sperm were already produced and when they subsequently scored male fertility only 9 days *post coitus* – a time frame that may not be sufficient for sperm depletion.

By investigating the effects of DNMT1 in males of *O. fasciatus*, I hope to not only show a conserved function between the sexes of this species but also introduce the idea of that function being conserved across males of other species. In doing so, knowledge of spermatogenesis and testis development in *O. fasciatus* would be invaluable.

Spermatogenesis and Testis Development in *Oncopeltus fasciatus*

Oncopeltus fasciatus have paired testes in the male reproductive system. Each testis is covered by a yellow sheath in which seven testis follicles are encased. Serving as the base unit of the testis, a testis follicle is the site where spermatogenesis occurs. Within the testis follicle are three cell types: follicle border cells (FBCs), apical cells, and germline stem cells (GSCs) (Schmidt et. al 2002), as *Figure 1.1* illustrates. Follicle border cells are evenly dispersed throughout the testis follicle. However, when migrating to its apical region, FBCs are now termed cyst progenitor cells. Apical cells form a complex always confined to the apical region of the testis follicle. The function of apical cells centers around the nourishment of GSCs: the cells outlining the apical cell complex. However, a regulatory function has been proposed. It has been hypothesized that apical cells signal GSCs to maintain their identity (Schmidt et. al 2001), and thus act as the stem cell niche. GSCs have projections connecting them to apical cells, but once these projections undergo apoptosis, they dissociate from the apical cells and subsequently divide to form spermatogonia. Nevertheless, whether the function of apical cells is trophic or regulatory, it is still an important contribution to spermatogenesis. As for GSCs, they can undergo symmetrical division and stay in close association with the apical cell complex or they can undergo asymmetrical division to form spermatogonia. If the latter, a cyst progenitor cell will start to encase a spermatogonium. Once that process is complete, the cyst progenitor cell is now termed *cyst* while the spermatogonium and the rest of its progeny are now termed *spermatocytes*. A cyst and its spermatocytes are collectively known as *spermatocysts*. Once spermatocytes proliferate to a number of 16, the onset of meiosis occurs (Schmidt et. al 2002). Transitions from spermatogonia to primary spermatocytes, secondary spermatocytes, spermatids, and eventually spermatozoa are housed within cysts. During these transitions, spermatocysts move toward the basal end of the testis follicle and are released into the seminal vesicles for storage.

As larvae in their first instar (L1), approximately four GSCs outline 12 apical cells in the distal (apical) region of the testis follicle (Schmidt et. al 2001). Beginning in L1 and throughout adulthood, apical cells are quantitatively fixed while GSCs are not. In L1 and the former half of L2, GSCs undergo symmetrical division to increase their numbers. From the latter half of L2 to the end of adulthood, GSCs asymmetrically divide to give rise to spermatogonia. Simultaneously, in the L2 stage, FBCs are migrating to the distal region of the cell to become cyst progenitor cells. At the start of L3, six spermatocytes are present, but their numbers increase during L4 and L5. By the end of L5, more than 50 spermatocytes have been observed. However, spermatids and spermatozoa aren’t observed until L4 and L5, respectively. By the end of L5, spermatozoa occupy approximately 50% of the testis follicle while the other 50% largely consists of less differentiated germ cells like spermatocytes, spermatogonia, and GSCs. After the imaginal molt, spermatozoa are released into and stored within the seminal vesicles.

Associated with these changes is the overall growth of the testis. The size and shape of the testis follicles change as a result of spermatocyst formation (Schmidt et. al 2001). L1 testis follicles are only 42 micrometers, but they grow to be 1,030 micrometers upon adult emergence. Even into adulthood, the volume of the testes can increase by 2% (Economopoulos and Gordon 1971). Regarding shape, L1 and L2 testis follicles are globular, but as spermatocytes form, the follicles increasingly become tubelike throughout the rest of development.

Predictions

I hypothesized that DNMT1 is required for germ cell development in *O. fasciatus***.** Given the oogenic compromise resulting from an absence of DNMT1 in females, I sought to identify any similar gametogenic effects in males. Three predictions stemmed from this hypothesis and with my knowledge of spermatogenesis and testis development, I sought to accurately test those predictions.

Prediction 1: The function of DNMT1 is conserved across the sexes.

Both males and females of *O. fasciatus* undergo meiosis. If DNMT1 is required for meiosis, then I predicted that both male and females will have similar gametogenic effects.

Prediction 2: DNMT1 is required during sexual development.

Sexual development is marked by the major onset of meiosis, but reports about the stage in which it's initiated are not in agreement with each other. Economopoulos and Gordon report meiotic activity begins in the L5 stage while Schmidt et. al report the presence of spermatids in the L4 stage (Economopoulos and Gordon 1971, Schmidt et. al 2002). Furthermore, in the L4 stage, the volume of the testes increases five-fold – specifically 50-75% every day (Economopoulos and Gordon 1971). Contra, the volume of L5 testis follicles only grows by 20% each day. In remembering growth is associated with meiotic activity, this, in combination with the presence of spermatids in L4s, led us to more reasonably conclude L4 as the stage in which the major onset of meiosis occurs. In doing so, we treated larvae before and after the major onset of meiosis in order to observe the impact on sexual development. If DNMT1 is required for sexual development, then I predicted the absence of DNMT1 would have a more negative impact in a premeiotic larval stage compared to a post-meiotic one.

Prediction 3: DNMT1 is required for sperm replenishment.

During copulation, spermatozoa are depleted from the seminal vesicles. Because males continue to produce sperm throughout their adulthood, spermatogenesis and meiosis are still necessary. If spermatogenesis stems from the presence of DNMT1, then I predict its absence will lead to a failure to replenish depleted sperm reserves.

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CHAPTER 2

THE ESSENTIAL ROLE OF DNMT1 IN GAMETOGENESIS IN THE LARGE MILKWEED BUG, *Oncopeltus fasciatus*

INTRODUCTION

Despite the apparent ubiquity of DNA methylation across the eukaryotic tree of life (Schmitz et al. 2019, Lewis et al. 2020), in insects, there is a considerable amount of variation both in the presence and extent of DNA methylation and even the presence and number of the DNA methyltransferases (Bewick et al. 2016, Lyko 2018, Glastad et al. 2019). Thus, the functional role of DNA methylation and its associated DNA methyltransferases in the insects is unclear. While some studies have associated levels of methylation with gene expression, most studies in the insects find no functional association (Bewick et al. 2016, Glastad et al. 2019).

One role emerging from knockdown and silencing studies across several insect species is that Dnmt1 is required for oogenesis (Schulz et al. 2018, Bewick et al. 2019, Gegner et al. 2019, Amukamara et al. 2020). For example, Dnmt1 is required for maintenance of DNA methylation following cell division in the milkweed bug, *Oncopeltus fasciatus* (Bewick et al. 2019, Amukamara et al. 2020), the downregulation of Dnmt1 using RNAi results in a reduction in methylation and also the cessation of oogenesis. However, it is unclear whether the effect on oogenesis is mediated by the reduction in DNA methylation (Amukamara et al. 2020). While the expected reduction in DNA methylation is seen throughout the organism following Dnmt1 knockdown, the only phenotypic consequence is to the germ cells. Evidence from other species supports a function independent of DNA methylation. Dnmt1 is also essential to egg production in T. castaneum (Schulz et al. 2018), a beetle that has no DNA methylation at all (Zemach et al.

2010). This suggests that Dnmt1 can have a function specific to germ cells in insects that is independent of its function in maintaining DNA methylation. This led us to hypothesize that Dnmt1 plays a role in meiosis in insects (Amukamara et al. 2020).

In this study, we tested the hypothesis that Dnmt1 is essential to gametogenesis in *O. fasciatus.* Furthermore, if Dnmt1 has a role in gametogenesis, including meiosis, it should be conserved across the sexes. Testing the function of Dnmt1 in males allows us to capitalize on the well-characterized process of spermatogenesis in *O. fasciatus*, specifically (*Figure 1.1,* Economopoulos and Gordon 1971) and the conserved features of insect spermatogenesis, generally (Dallai 2014). In *O. fasciatus*, as in many insects, there are two points in development where meiosis can occur. The first stage where meiosis will occur is during the larval stages. Testis development and spermatogenesis is initiated during larval development in *O. fasciatus* (Economopoulos & Gordon 1971). During the first three instars the testes consist of seven globular follicles that will develop into the testis tubules (Schmidt et al. 2002). Meiosis is initiated in the 4th instar, and by the end of the 4th instar, cysts containing spermatids are present. Differentiation of the spermatids commences in the 5th instar, and males emerge with up to 250,000 spermatids that continue to differentiate during sexual maturation. Thus, we can target the developmental time point at which meiosis occurs. In addition, *O. fasciatus* males can produce gametes throughout their adult lives and therefore meiosis can occur in the adult testis. If Dnmt1 is required for meiosis and gametogenesis we predicted that it will be required both during larval development and as adult males replenish sperm stores following mating. We therefore compared the testis phenotypes of adults developing from nymphs in which Dnmt1 expression was downregulated either before or after the major wave of meiosis that occurs in testis development (Economopoulos & Gordon 1971, Schmidt et al. 2002, Ewen-Campen et al 2013). We also treated sexually mature adults, and examined fertility in males following sperm depletion, testing for the ability of knockdown males to replenish sperm stores following multiple matings. Our results demonstrated that expression of Dnmt1 is required for the development of sperm during both larval and adult spermatogenesis and that the impact of Dnmt1 knockdown was greatest if it occurred prior to the onset of meiotic divisions in the developing testes. These results establish that Dnmt1 plays a critical conserved function across the sexes during gametogenesis in *O. fasciatus* and that it is required for germ cell development.

RESULTS

The pattern of Dnmt1 expression during testis development and across tissues suggests a role in spermatogenesis.

We measured Dnmt1 expression during larval development and sexual maturation. Our prediction was that if Dnmt1 is required during gametogenesis, particularly meiosis, then its expression should mirror those of two genes known to be involved in germ cell development, *boule* and *vasa* (Shah et al. 2010, Yajima & Wessel 2011). Thus, we predicted that Dnmt1 expression would be highest during the stages when gametogenesis is occurring. As predicted, testis-specific expression of Dnmt1 is highest during the 4th and 5th instars of development (*Figure 2.1A*; ANOVA, F = 27.426, d.f. = 3, 38, $p < 0.001$), stages when meiosis and spermatogenesis are most active (Economopoulos & Gordon 1971). The expression is lowest in virgin adult males. This expression pattern mirrored that observed for Boule, a gene with a well-characterized role in meiosis (*Figure 2.1B*; ANOVA, F = 36.346, d.f. = 3, 38, $p < 0.001$). Dnmt1 expression pattern was also similar to Vasa, a highly conserved marker of the germline (*Figure*

2.1C; ANOVA, F = 20.444, d.f. = 3, 38, p < 0.001). While Dnmt1 is expressed across all adult tissues, as would be expected given its role in maintenance of DNA methylation, expression is highest in testes compared to gut, head, and muscle tissue (*Figure 2.2*; ANOVA, F = 67.311, d.f. = 3, 36, p < 0.001), also suggesting a special role during gametogenesis.

Dnmt1 knockdown reduced levels of DNA methylation in the testis genome.

To confirm the effectiveness of our RNAi treatment, we measured expression of Dnmt1 in a subset of ds-RED and ds-Dnmt1 injected males (*Figure 2.4*). Treatment of males with ds-Dnmt1 had the predicted effect on Dnmt1 expression. We performed whole genome bisulfite sequencing to evaluate the impact of the ds-Dnmt1 on DNA methylation genome wide. The reduction in expression of Dnmt1 in the RNAi individuals had the expected phenotypic effect on DNA methylation with a reduction of genome methylation in the testes for ds-Dnmt1 treated males, but not control males (*Figure 2.3*). Genomic DNA from the testes of control treated males had approximately 12.5% CpG methylation regardless of stage they were treated. Knockdown of Dnmt1 at the earlier stage of development led to a greater percentage reduction of methylation. Treatment with ds-Dnmt1 at the 5th instar reduced the percent CpG methylation from around 12.5% to around 5%. Treatment with ds-Dnmt1 at the 3rd instar reduced the level of methylation even further, to around 2%, as predicted given the greater numbers of cell divisions that were expected between treatment and sampling between these two treatments.

Dnmt1 knockdown affected testis size and structure.

Knockdown of Dnmt1 prior to meiosis, in the 3rd instar stage, had a significant effect on testis size in virgin males, while knockdown of Dnmt1 during the developmental stage after which meiosis is initiated, the 5th instar stage, had no effect on testis size (*Figure 2.5*; ANOVA, F = 20.360, d.f. = 4, 58, $p < 0.001$) compared to either uninjected controls or ds-RED treated males. Knockdown of Dnmt1 prior to meiosis affected testis tubules to a greater extent than knockdown following the initiation of meiosis during larval testis development (*Figure 2.6*). Control males injected in the 3rd or 5th instar stage of development showed the expected structure of the testis tubule (Figure 2.6A and C). At the anterior end of the testis tubule, spermatogonia and primary spermatocytes had the characteristic nuclear structure and there was clear evidence of mitotic division within the spermatogonia (Figure 2.6C, arrowhead), using α -phosphohistone H3 (pHH3) to stain for chromosome condensation in preparation for mitosis and meiosis. We also observed α-pHH3 staining in spermatocysts at the border between primary and secondary spermatocytes in the control testis tubules in both 3rd and 5th instar treated males. Following this band of relatively synchronous meiotic activity, the posterior testis tubule showed spermatids and developing spermatozoa as they matured. We confirmed the use of this band of α-pHH3 stained spermatocysts as a landmark of meiosis using knockdown of Boule (*Figure 2.7*).

The structure of the testis tubules of males treated with ds-Dnmt1 in the 3rd instar was highly disrupted and both the anterior and posterior testis tubule was affected (*Figure 2.6B*). There were fewer spermatocysts in both the region of spermatogonia and primary spermatocytes. While there were occasional α-pHH3 positive nuclei, these were not well organized into spermatocysts and were spread throughout the testis tubule rather than organized at the junction between primary and secondary spermatocytes. There was evidence of mitotic activity in the spermatogonia (*Figure 2.6B* arrowhead), although these were less frequent in ds-Dnmt1 treated males than control males. The testis tubules of males treated with ds-Dnmt1 in the 5th instar stage of development had a structure much more similar to control males (*Figure 2.6D*). Mitotic activity was apparent in the spermatogonia (*Figure 2.6D* arrowhead) and most testis tubules had evidence of mature sperm and α-pHH3 stained spermatocysts at the junction between primary and secondary spermatocytes (*Figure 2.6D* arrow). Unorganized spermatocysts below this junction were frequently observed, however, and ds-Dnmt1 males treated at the 5th instar had variable phenotypes posterior to the primary spermatocytes, presumably depending on when treatment occurred following the wave of meiosis along the testis tubule axis.

Dnmt1 knockdown in adult males prevented replenishment of sperm stores

Downregulating Dnmt1 expression in adult males resulted in a loss of fecundity over time. The third and final females mated to ds-Dnmt1 treated males ran out of sperm to fertilize eggs more rapidly than those mated to control males. Clutches of eggs laid by females mated to ds-Dnmt1 treated males were not fertilized and failed to hatch at an earlier collection day than those laid by females mated to control males (*Figure 2.8;* Wilcoxon $\chi^2 = 13.978$, d. f. = 1, $p < 0.001$).

The loss of fertility in males was associated with smaller testis size. Males treated with ds-Dnmt1 at seven days post-emergence and then allowed to mate for three weeks prior to dissection had statistically significantly smaller testis area than the testes of control males after the same mating treatment (ANOVA, F = 29.084, d.f. = 1, 51, $p < 0.001$; *Figure 2.9*). The smaller testis area of ds-Dnmt1 treated adult males was associated with a breakdown in testis tubule structure and the loss of sperm from the testis tubules (*Figure 2.10*). In control males, three weeks post injection the regions of spermatogenesis were recognizable. Small spermatocysts with spermatogonia undergoing mitotic divisions were observable at the anterior tip of the testis tubule (*Figure 2.10A* and *B*). Posterior to the spermatogonia were the primary spermatocytes with more diffuse nuclear structure. We often observed the band of α-pHH3 positive spermatocysts that indicated the first meiotic division to form the secondary spermatocytes. At the most posterior end of the testis tubule the spermatids developed into spermatozoa. Three weeks after Dnmt1 knockdown testis tubule structure is significantly disrupted. There appeared to be fewer spermatogonia and those that remained had a more condensed nuclear structure than in the testis tubules of control males (*Figure 7C* and *D*). The most noticeable effect was seen in the region containing the spermatocytes. There were few primary spermatocytes in the testis tubule and α-pHH3 positively stained spermatocysts were rarely observed, indicating few spermatocysts undergoing meiosis.

Discussion

The function of DNA methylation and the DNA methylation enzymes across the insect tree of life have been widely debated, particularly in light of the extreme variation in presence of this chromatin modification and the diversification of the enzymatic toolkit required to methylate DNA de novo and maintain DNA methylation patterns (Bewick et al. 2016, Lyko 2018, Lewis et al. 2020). This evolutionary pattern is particularly intriguing given that in two species on divergent branches of the insect phylogeny the maintenance methyltransferase DNMT1 is essential to gametogenesis, and thus is tightly tied to fitness, even in a species where DNA methylation itself is absent (Schulz et al. 2018). While functional studies have not been done in other species, a summary of expression studies on the DNA methyltransferases across a diversity of insects suggest that these enzymes could be involved in gametogenesis across a number of species, ranging from fire ants, *Solenopsis invicta* (Kay et al. 2018), to jewel wasps, *Nasonia vitripennis* (Zwier et al. 2012), to migratory locusts, *Locusta migratoria* (Robinson et al. 2016), and the brown plant hopper, *Nilaparvata lugens* (Zhang et al. 2015). We showed that Dnmt1 expression affects germ cell development. The effect of downregulating Dnmt1 in males, as in females (Amukamara et al. 2020), was specific to the germ cells. The downregulation of Dnmt1 did result in reduction of DNA methylation, but there were no obvious morphological impacts to the RNAi-treated individuals. Our results show that the function of Dnmt1 in germ cells is conserved across the sexes within a species, and other results document that Dnmt1 expression is required for oogenesis among species. This raises a conundrum when we look across the insect tree of life. How can a gene required for such a fundamental fitness activity in examples from such a diverse group of insects also be so evolutionarily labile? This suggests that the function Dnmt1 in germ cell development is easily lost or replaced.

Dnmt1 knockdown causes inhibition of spermatogenesis consistent with a role in meiosis

If our hypothesis on the role of Dnmt1 in meiosis is correct, then spermatogenesis should be affected by Dnmt1 expression as well as oogenesis, as it is a shared process across gametogenesis during sexual reproduction. In the testes *O. fasciatus* males, we observed both spermatogonia and primary spermatocytes that stain positively for phosphorylation of the serine 10 residue of histone protein H3. While the pattern of histone H3 phosphorylation across meiosis has not been specifically studied in *O. fasciatus*, phosphorylation of the serine 10 is typically associated with chromosome condensation during meiosis (Hans and Dimitrov 2001) and mitosis (Prigent and Dimitrov 2003). Moreover, the pattern of α-pHH3 staining along the axis of the testis tubule is consistent with this histone modification occurring in both mitosis and meiosis. We observed α-pHH3 positive cells both within spermatocysts containing the mitotically dividing spermatogonia and also at the boundary between primary and secondary spermatocytes. We have confirmed this landmark using Boule knockdown males. In *Drosophila*, Boule is required for progression of spermatocytes through meiosis. Germ cells lacking functional Boule become arrested at meiotic prophase (Eberhart et al. 1996). In the testes of *O. fasciatus* males treated with ds-Boule, the affected cells were all posterior to the band of synchronous α-pHH3 positive spermatocytes. Thus α-pHH3 staining provided a landmark for entry into meiosis.

We did not see the band of α-pHH3 positive spermatocysts at the border between primary and secondary spermatocytes in the Dnmt1 knockdown males treated at the 3rd instar stage of development. This could be interpreted as the primary spermatocytes have not initiated chromosome condensation. However, we did observe individual α-pHH3 positive nuclei in the posterior testis tubule. So there could have been arrest after chromatin condensation with a breakdown in spermatocyst structure. Interestingly, the testis phenotype of our Dnmt1 knockdown males resembled the phenotypic effect of Vasa knockdown reported by Ewen-Campen et al. 2013. Vasa RNAi results in defects in cyst integrity and Ewen Campen et al. (2013) propose that Vasa plays a specific role in the onset or synchrony of meiosis. Vasa has also been proposed to be required for the correct progression through meiosis in mice (Tanaka et al. 2000) and humans (Medrano et al. 2012). Thus, the phenotypic similarity between Dnmt1 knockdown and Vasa knockdown testes in *O. faciatus* support the hypothesis that Dnmt1 may be required for the successful initiation and completion of meiosis in spermatocytes. More work remains to determine exactly what stage of sperm development is impacted by the decrease in Dnmt1 expression.

The phenotype of knockdown of Dnmt1 is exacerbated when knockdown occurs prior to the stage of testis development when meiosis occurs. This result is consistent with what was observed in female *O. fasciatus* (Amukamara et al. 2020) in which knockdown of Dnmt1 expression prior to the stage of oocyte development inhibited all oocyte formation. One interpretation of this result is that Dnmt1 is required for successful progression through meiosis. However, it is clear that reducing Dnmt1 expression had an impact beyond simply reducing the ability to enter or complete meiosis. A block to progression through meiosis, as demonstrated by the Boule knockdown, spermatogonia and primary spermatocytes would continue to be born but remain viable after a failure to complete meiosis. In the Dnmt1 knockdowns, however, there were fewer germ cells of any type, and those that remained in the testis tubule often appeared to have abnormal and condensed nuclei. It remains to be determined if Dnmt1 is required for meiosis or if the association with meiosis is a correlation; Dnmt1 could be required for viability of germ cells and act at the stage of development at which they would be undergoing meiosis.

Previous studies on the function of Dnmt1 in insect spermatogenesis have not documented any effect on male fertility. Knockdown of Dnmt1 in the red flour beetle*, T. castaneum* (Schulz et al. 2018) had no effect on the fecundity of female mating partners was measured in the first nine days post mating and the authors conclude that Dnmt1 is not required for spermatogenesis in *T. castaneum*. We suspect that the function of Dnmt1 in spermatogenesis is not unique to O. fasciatus, given the expression patterns of Dnmt1 in other insects (Robinson et al. 2016, Kay et al. 2018). In the *T. castaneum* study, the authors did not examine testis structure or design a mating strategy that would exhaust the sperm stores of the males and so may have missed a fertility effect. In holometabolous insects, including beetles, spermatocytes are typically formed by the end of larval development (Economopoulos & Gordon 1971), thus males treated as pupae would still emerge with large numbers of sperm in their testes. Here we showed that male *O.*

fasciatus treated with ds-Dnmt1 RNA as sexually mature adults become sperm limited more rapidly than control males. There is a potential alternative explanation, that Dnmt1 knockdown males could continue to produce sperm but that sperm was of low quality and unable to support development. Given that the testes of ds-Dnmt1 treated males were significantly smaller than control males and had altered testis tubule structure, which indicated that spermatogenesis was significantly impacted, we concluded that Dnmt1 knockdown males are unable to replenish sperm supplies. The functional role of Dnmt1 in testes across other species remains to be tested.

DNA methylation and germ cell development

The knockdown of Dnmt1 resulted in a reduction of DNA methylation within the testes, as predicted. The effect was greater in the nymphs that were treated in an earlier stage of development, as would be expected given the greater number of cell divisions that would occur between treatment and collection of the testes between the 3rd and 5th instar treatments. The more extreme phenotype in the 3rd instar treated males could be explained by the greater reduction in methylated CpG. However, the percent methylation seen in the testes of the 5th instar ds-Dnmt1 treated males was similar to that seen in females treated at the 4th instar stage of development (Amukamara et al. 2020), greater than a two-fold reduction in the percent methylated CpG residues. In Dnmt1 knockdown females, there was a compete loss of oocyte production when DNA methylation was reduced to this level while in males the phenotype of the 5th instar Dnmt1 knockdowns was close to normal. This disconnect between reduction of DNA methylation and phenotypic effects mirrors what has been seen in the beetle, *T. castaneum* (Schulz et al. 2018). In this species, the reduction of DNA methylation is an evolved difference rather than an experimental effect, but the results are similar to what we have observed in *O.*

fasciatus; DNA methylation is not required for function of somatic cells, but downregulation of Dnmt1 expression leads to specific germ cell effects. The lack of correlation between extent of methylation and function in germ cells strengthens our hypothesis that there may be a pleiotropic function for Dnmt1 in germ cells that acts independently of DNA methylation levels.

Alternatively, DNA methylation may have a specific role in the germ cells that it does not play in somatic cells. Perhaps DNA methylation is required for proper gene expression in germ cells or it may be required for transposon silencing only in germ cells. However, demethylation of ovarian tissue in *O. fasciatus* showed little relationship between methylation levels and gene expression, with few differentially transcribed genes (Bewick et al. 2019). Another possibility is that DNA methylation state may be a prerequisite for successful completion of meiosis and not mitosis. For example, O. fasciatus has holocentric chromosomes and undergoes inverted meiosis (Viera et al. 2009). During mitosis chromosomes have a kinetocore that extends across the majority of the chromosome but in meiosis, the kinetochore plate is missing and microtubules extend into the chromosome (Comings & Okada 1972). One could imagine that DNA methylation might be required for this interaction. However, the complete lack of methylation but a functional role of Dnmt1 in *T. casteneum* oogenesis alongside our results argues for a pleiotropic function for Dnmt1 in gametogenesis that is independent of its role in DNA methylation.

Materials and Methods

Animal care

All experimental animals were from colonies of laboratory reared *O. fasciatus* (Carolina Biologicals, Burlington, NC) and were reared under standard rearing conditions of 12 hr:12 hr light/dark at 27□ C. To collect animals of known age and social conditions, eggs were removed from the mass colonies and allowed to hatch in plastic storage containers containing ad libitum deionized water and organic, raw sunflower seeds. For the nymph injections, nymphs were pulled from mixed sex nymph colonies at the 3rd instar or 5th instar. For adult injections, nymphs were separated by sex at the 4th instar and housed in single sex colonies. These were checked daily and newly emerged adults. All experimental animals were placed into individual petri dishes with food and water.

Developmental expression

Given our hypothesis that Dnmt1 is required for development of germ cells and meiosis we examined the expression levels of Dnmt1 across testis development in males. Groups of nymphs were staged and sexed. Testes were dissected from 4th and 5th instar nymphs and flash frozen in liquid nitrogen and stored at -80℃. We also collected testes from virgin males on the day of adult emergence and after sexual maturation at 7 days post-adult-emergence. We also collected head, gut, and muscle samples from the sexually mature adult male individuals for tissue specific expression.

Total RNA was extracted using a Qiagen RNA easy kit with Qiazol (Qiagen, Venlo, The Netherlands) and complementary DNA (cDNA) synthesized from 500 ng RNA with aScript cDNA Super-Mix (Quanta Biosciences, Gaithersburg, MD). Quantitative real-time PCR (qRT-PCR) was used to determine expression levels of Dnmt1 and two genes with known functions in spermatogenesis, Boule and Vasa. Primers are described in Amukamara et al. 2020. As in Amukamara et al. 2020, actin and GAPDH were used as reference genes. We have previously validated these reference genes and they are accepted as robust reference genes in O. fasciatus (Meinzer et al. 2019). We used a Roche LightCycler 480 with the SYBR Green Master Mix (Roche Applied Science Indianapolis, IN). All samples were run with 3 technical replicates using 10 μL. There were 10 biological replicates for each stage. Each biological replicate of 2nd, 3rd, 4th, and 5th instar nymphs consisted of pools of 10, 5, 4, and 3 individuals, respectively. Adult replicates consisted of individual animals. Sample size was based on past experience balanced by the cost of qRT-PCR. Primer efficiency calculations, genomic contamination testing, and endogenous control gene selection were performed as described in Cunningham et al. 2014. We used the delta-delta CT method to compare levels of gene expression across the samples (Livak and Schmittgen 2001). Gene expression was standardized per individual to account for different numbers of individuals within each group at each developmental stage. Differences in expression levels were analyzed using ANOVA in JMP Prov14. If there was a significant overall effect, we compared means using Tukey-Kramer HSD.

RNAi preparation

Double-stranded RNAs were prepared as described in Amukamara et al. 2020. Briefly, DNA templates were prepared by PCR using gene-specific primers (Amukamara et al. 2020). Sense and anti-sense RNA were transcribed together with an Ambion MEGAscript kit (ThermoFisher Sci, Waltham, MA) and allowed to anneal to form a 404 bp ds-Dnmt1 RNA. The concentration of dsRNA was adjusted to 3 μg/μL in injection buffer (5 mM KCl, 0.1 mM NaH2PO4).

Nymph injections, testis size and morphology

To examine the effect of Dnmt1 knockdown prior to or following the wave of meiosis initiated in the 4th instar stage, nymphs were injected with ds-Dnmt1 or control ds-RED injections at either the 3rd instar or 5th instar stage of development. For further information on controls and testing for potential off-target effects please see Amukamara et al. 2020. Nymphs were anaesthetized at 4 degrees C for 20 minutes prior to injection. Nymphs were injected in the abdomen using pulled glass capillary needles (Sutter Instrument Company micropipette puller model P-97, Novato, CA) between the third and fourth abdominal segments (Chesebro et al. 2009). Nymphs were injected with 2 µL volume for all injections. Following injections, nymphs were placed in individual petridishes and monitored for development. Date of adult emergence was recorded. We did not do a power analysis, but based on preliminary data on the strength of the effect we aimed for 25 individual males for each treatment. Males were randomly assigned to a treatment group. Not allmales survived to age of dissection, resulting in final sample sizes of 22 and 19 for 3rd instar males injected with buffer and ds-Dnmt1, respectively and 25 and 20 for 5th instar males injected with buffer and ds-Dnmt1, respectively.

Testis size: At 7 to 10 days post-adult emergence, virgin males were dissected and their testes were removed. Whole testes were allowed to settle into 1 mL Phosphate Buffered Saline (PBS) and were imaged with a Leica M60 Stereomicroscope with Leica Application Suite software (LAS v4). Testis area was measured on one of the pair from each male with the LAS by outlining the whole testis with all 7 testis tubules. Differences in testis area were analyzed using ANOVA in JMP Pro v14.

Testis tubule structure: *O. fasciatus* testes contain 7 individual testis tubules surrounded by a relatively impermeable, autofluorescent membrane. Individual testis tubules were removed from the outer membranous sheath for fixation and staining. Males from each treatment were dissected across 4 dissection days. Testis tubules from individual males within a treatment were pooled for staining. Thus each day one tube was a replicate with tubules from several individual males. Tubules were fixed for 30 minutes in 4% formaldehyde in PBS plus 0.1% Triton-X100 (PBT) and stained for evidence of cell division using an α -phosphohistone H3 Ser10 (pHH3) primary antibody (Millipore antibody 06-570, Sigma-Aldrich, St. Louis, MO). α -phosphohistone H3 (pHH3) stains for chromosome condensation in preparation for mitosis and meiosis (Hans & Dimitrov 2001, Prigent & Dimitrov 2003). The secondary antibody was an Alexa Fluor goat-anti-rabbit 647 (ThermoFisher Scientific, Waltham, MA). Following antibody staining the tubules were stained with DAPI (0.5μg/mL PBT) to visualize nucleic acids. Stained tubules were mounted in Mowiol 4-88 mounting medium (Sigma-Aldrich, St. Louis, MO) and visualizedwith an Olympus BX51 Fluorescent microscope. Images were taken of every testis tubules present on each slide. Representative images are presented in the figures.

Quantitative Real Time PCR

While we had evidence that all our RNAi treatments successfully knocked down expression in females (Amukamara et al. 2020), to confirm that our RNAi treatment was effective in males, total RNA and genomic DNA was extracted from flash frozen testes of a subsample of males 6 to 10 days following injection with the dsRNA using a Qiagen Allprep DNA/RNA Mini Kit (Qiagen, Venlo, The Netherlands). Expression levels for Dnmt1 was analyzed using qRT-PCR as described above.

Quantification of DNA methylation

The DNA from each prep used for qRT-PCR was used to prepare MethylC-seq libraries as described in Urich et al. 2015 and Amukamara et al. 2020. Samples were sequenced on a NextSeq500 and qualified reads were aligned to the O. fasciatus genome assembly according to previously published methods (Schmitz et al. 2013). The percent DNA methylation was calculated by dividing the total number of methylated CpG sites by the total number of CpG sites (Schultz et al. 2012). Spiked in Lambda DNA, which is fully demethylated, was used as a control for the sodium bisulfite conversion rate of unmodified cytosines. Three individuals were sequenced for each treatment.

Adult injections

RNAi treatment: Sexually mature virgin males (7 days post-adult-emergence) were injected with 3 µL ds-Dnmt1 RNA or control injections using a pulled glass capillary needle between the third and fourth abdominal segments (Chesebro et al. 2009). Control injections for the male fecundity experiments were injection buffer alone. We used ds-RED control injections for the testis imaging experiment. Previous studies from our lab have shown no difference in buffer alone controls or non-specific ds-RNA (Bewick et al. 2019). Following injection males were placed into individual petri dishes and provided with ad libitum food and water. We did not do a power analysis, but based on preliminary data on the strength of the effect we aimed for 30 individual males for each treatment. Males were randomly assigned to a treatment group.

Male fecundity: Preliminary experiments had shown that two previous matings were required to deplete sperm stores. Therefore, in order to allow males to deplete sperm stores acquired during nymphal development and sexual maturation, males were placed in mating trials with three 7- to 10-day-old virgin females, one provided each week. The first female was placed in the male's petri on the day of injection with cotton wool as an oviposition substrate. The female and all eggs were removed at the end of the week (7 days post-injection) and discarded and replaced with a second 7- to 10-day-old virgin female and fresh cotton wool. Again, at the end of the week (14 days post-injection), the female and all resulting eggs were discarded. A third, focal, 7- to 10-day-old virgin female was placed with the male with fresh cotton wool. The 3rd female and experimental male were given one week to mate before the male was removed from the petri dish for analysis of testis size and structure. The female was maintained for her lifetime, provided with ad libitum food and water. The eggs produced by the 3rd female were collected from the petri dish twice a week at 3-4 day intervals and the oviposition substrate replaced with fresh cotton wool. The eggs collected were placed in a separate container and allowed to develop to hatching (approximately 7-10 days following collection) and then frozen at -20^{o} C until assayed. We recorded the first collection date for which no eggs hatched. We analyzed the time to end of fertilized eggs due to sperm depletion relative to treatment using a survival analysis (Wilcoxon Rank Sum test) using JMP Pro v14.1. Not all males survived to date of dissection, or their mates died during the course of egg collection, requiring these males to be removed from the analysis, resulting in a sample size of 21 control and 20 ds-Dnmt1 treated males in the final fecundity analysis.

Testis size: At the end of the one-week mating trial with the 3rd female (21 days post-injection), males were dissected and their testes removed into 1 mL Phosphate Buffered Saline (PBS). Whole testes were photographed and measured as described above. Not all males survived to date of dissection, resulting in a final sample size of 28 control and 25 ds-Dnmt1 treated males. Testis tubule structure: Testis tubules from mated males were isolated, fixed and stained as described above.

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CHAPTER 3

CONCLUSION

By showing that DNMT1 has an essential role in gametogenesis in *Oncopeltus fasciatus*, I was able to advance the idea that DNMT1 has a function during gametogenesis in male insects. More interesting is the idea that this function is independent of methylation, as *T. castaneum* cannot reproduce without DNMT1 despite its undetectable levels of DNA methylation, suggesting a natural lack of a genome. Although our previous work shows a function exclusive to germ cells in *O. fasciatus*, we could not dissociate DNMT1 from DNA methylation within the germline. The reproductive phenotype resulting from downregulating DNMT1 could either mean that DNMT1 plays an essential role independent of DNA methylation during gametogenesis or that DNA methylation has a specific function within the germline that is not required in somatic cells. This entanglement also surfaced in my work. However, it can be disentangled if the genome remains methylated while DNMT1 is downregulated. As DNMT1 is one of the main enzymes known to methylate DNA, the challenge accounting for such a confounding variable lies in discovering an alternative mechanism responsible for DNA methylation. Hopefully, my thesis alongside other studies can kickstart that process in order to further substantiate the exclusive role DNMT1 plays in insect reproduction.

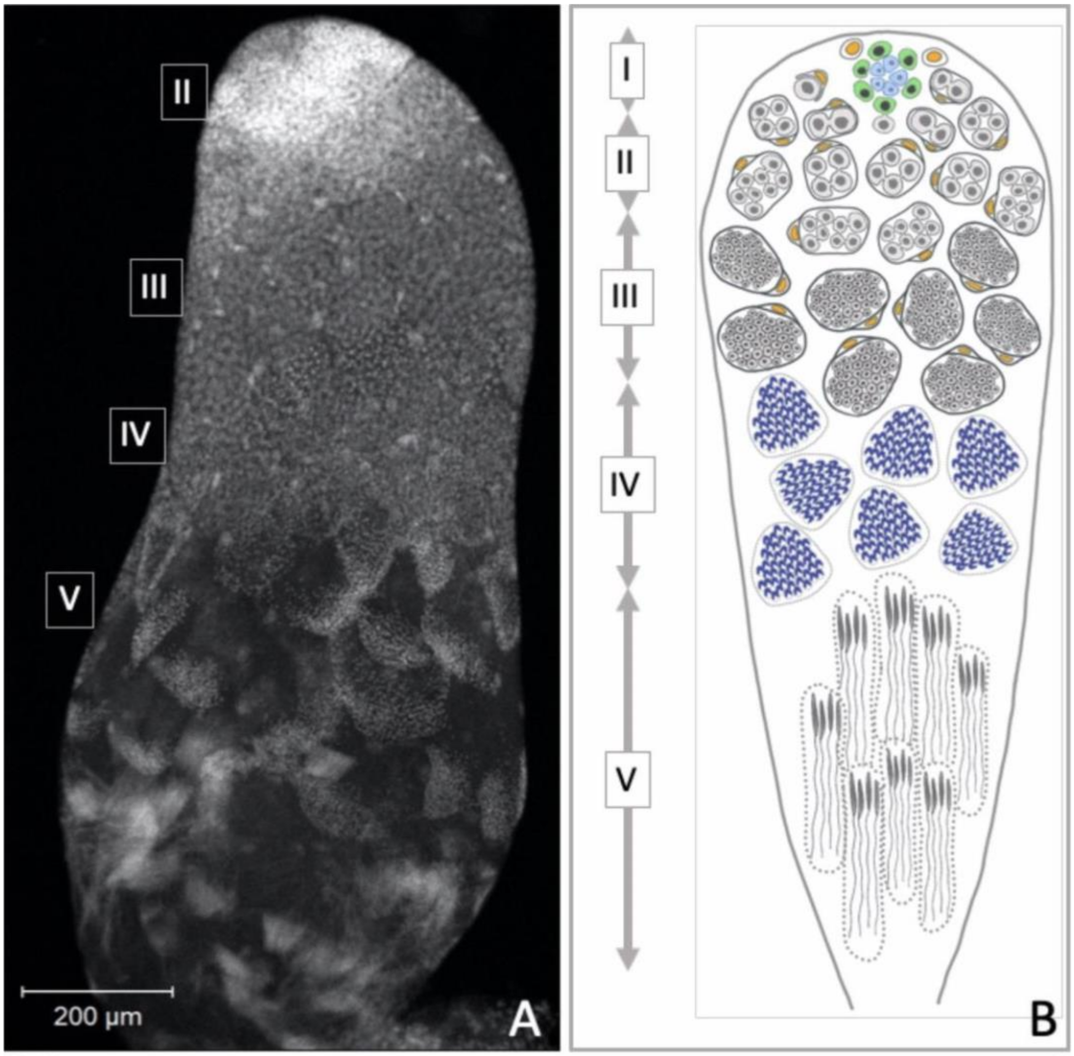

Figure 1.1 Progression of spermatogenesis in *O. fasciatus.* In *O. fasciatus*, spermatogenesis progresses from the tip of the testis tubule. (A) Dapi-stained testis tubule of stock male. (B) Diagram of the stages of spermatogenesis. At the apical tip (region I) of each of the seven testis tubules there is an apical stem cell complex (light blue) surrounded by the germline cells (B, green: Schmidt et al. 2002). The apical stem cell complex is not typically visible in confocal images of testis tubules and markers for the apical stem cell complex have not been identified yet. As spermatogonia (B, light gray) arise from divisions of the germline stem cells, they are enclosed by cyst cells (B, yellow). In region II, spermatogonia undergo mitotic transit amplification divisions to form spermatocysts containing 64 spermatogonia (Economopoulos and Gordon 1971). Ewen-Campen et al 2013). Spermatocytes (B, dark gray) in region III divide meiotically. *Oncopeltus fasciatus* undergoes inverted meiosis (Viera et al. 2009). Primary spermatocytes undergo the first meiotic division to produce diploid secondary spermatocytes. The meiotic division of the secondary spermatocytes produce the haploid spermatids (B, dark blue) in region IV that then differentiate into spermatozoa in the region V at the terminal end of the testis tubule.

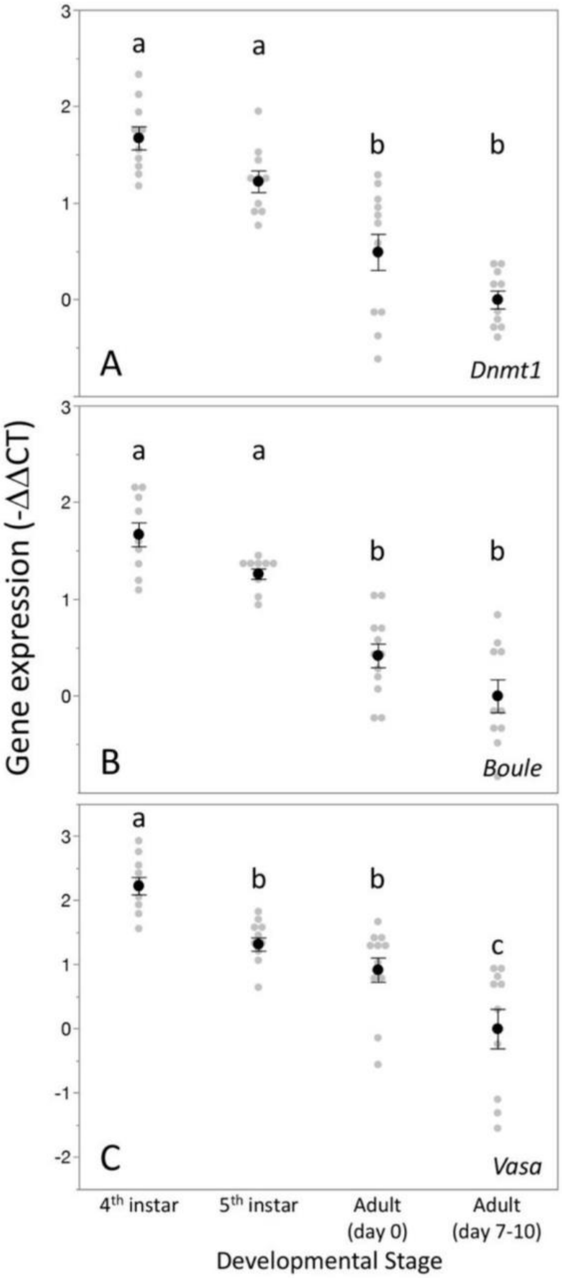

Figure 2.1 Expression pattern of Dnmt1 within the developing testes mirrored that the two gametogenesis genes Boule and Vasa. Dnmt1 expression (A) was highest in the larval stages where spermatogenesis is initiated compared to the testes of newly emerged (day 0) and virgin sexually mature (day 7-10) adults. This is similar to the expression patterns of Boule (B), in which expression was higher in the 4th and 5th larval stages during which the transition to meiosis occurs compared to newly emerged and virgin sexually mature adults. Expression of Vasa (C) demonstrated a similar pattern, with the highest expression in the 4th instar stage and lowest in sexually mature virgin males. Black dots and bars represent mean and SE. Grey dots represent data points for each individual tested. Lower case letters designate significant differences ($p < 0.05$) among pairwise comparisons using post-hoc Tukey-Kramer HSD test.

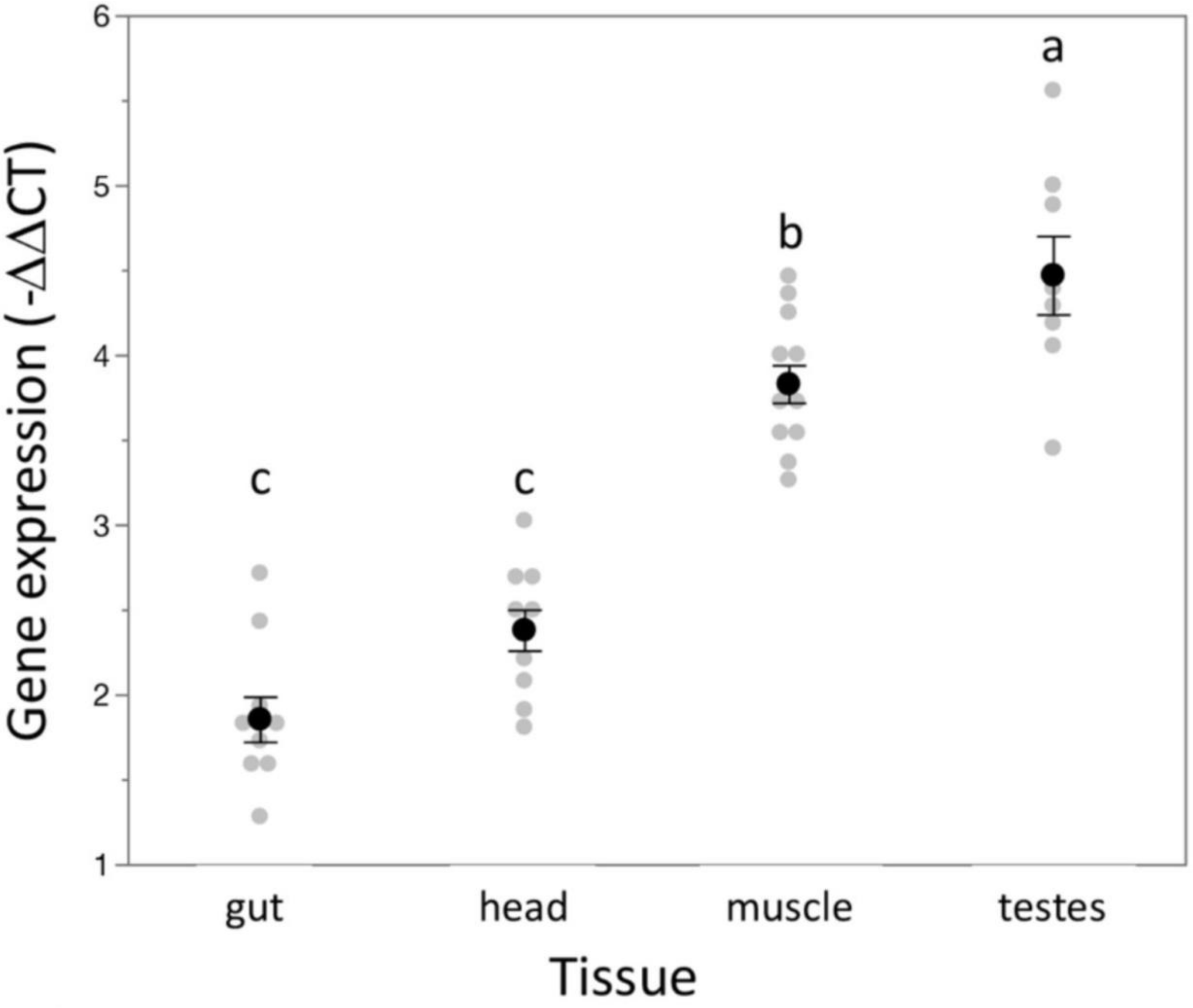

Figure 2.2 Expression of Dnmt1 is highest in reproductive tissue. As has been seen for females (Amukamara et al. 2020), expression of Dnmt1 varied across the type of tissue in males.. As in females, expression was lowest in the gut and head compared to the other somatic tissue tested, muscle. Expression is highest in the gonad (testes). Lower case letters designate significant differences ($p < 0.02$) among pairwise comparisons using post-hoc Tukey-Kramer HSD test.

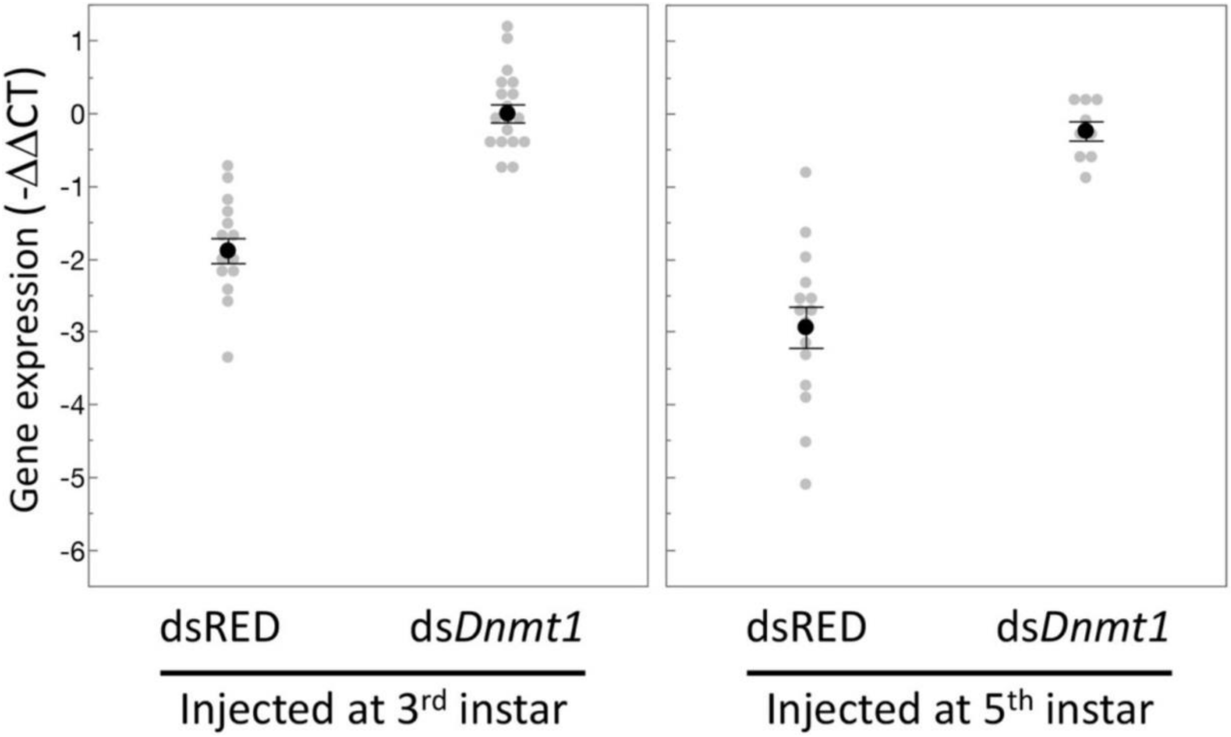

Figure 2.3 Expression of Dnmt1 was reduced in the testes of adult males treated with ds-Dnmt1 at both stages of development. Relative gene expression is standardized to expression levels in control treatments. Black dots and bars represent mean and SE. Grey dots represent data points for each individual tested.

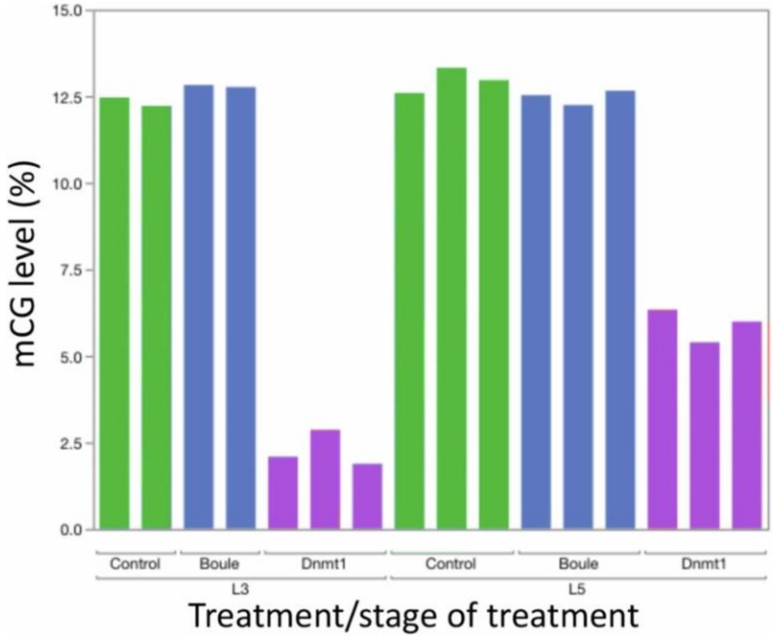

Figure 2.4 Knockdown of Dnmt1 (magenta bars), but not Boule (blue bars), reduced DNA methylation compared to controls (green bars). Each bar represents a single individual. Across all individuals, the percent methyl CpG is reduced in the DNA isolated from the adult testis of males treated with ds-Dnmt1. Earlier injection reduces the percent methylation to a greater extent, consistent with more rounds of cell division within the testes between injection and dissection

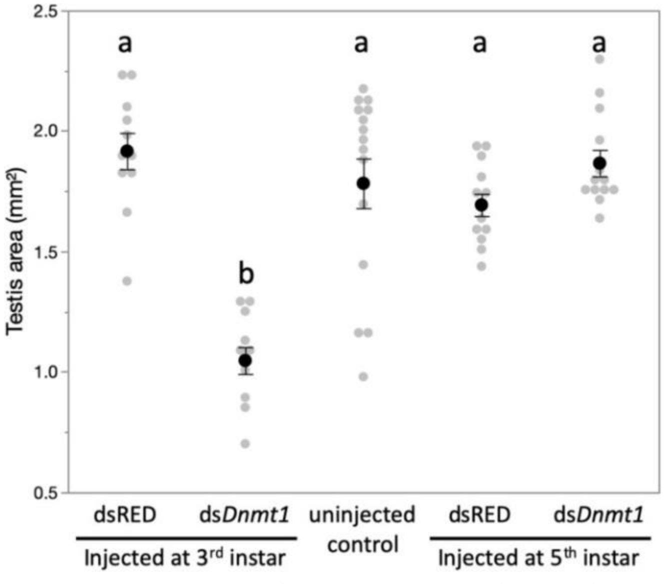

Figure 2.5 Downregulation of Dnmt1 during the 3rd instar stage of development significantly reduced the size of the testis in sexually mature males. There was no effect of downregulation of Dnmt1 on testis size when treatment occurred during the 5th instar stage of development after meiosis has been initiated. Black dots and bars represent mean and SE. Grey dots represent data points for each individual tested.

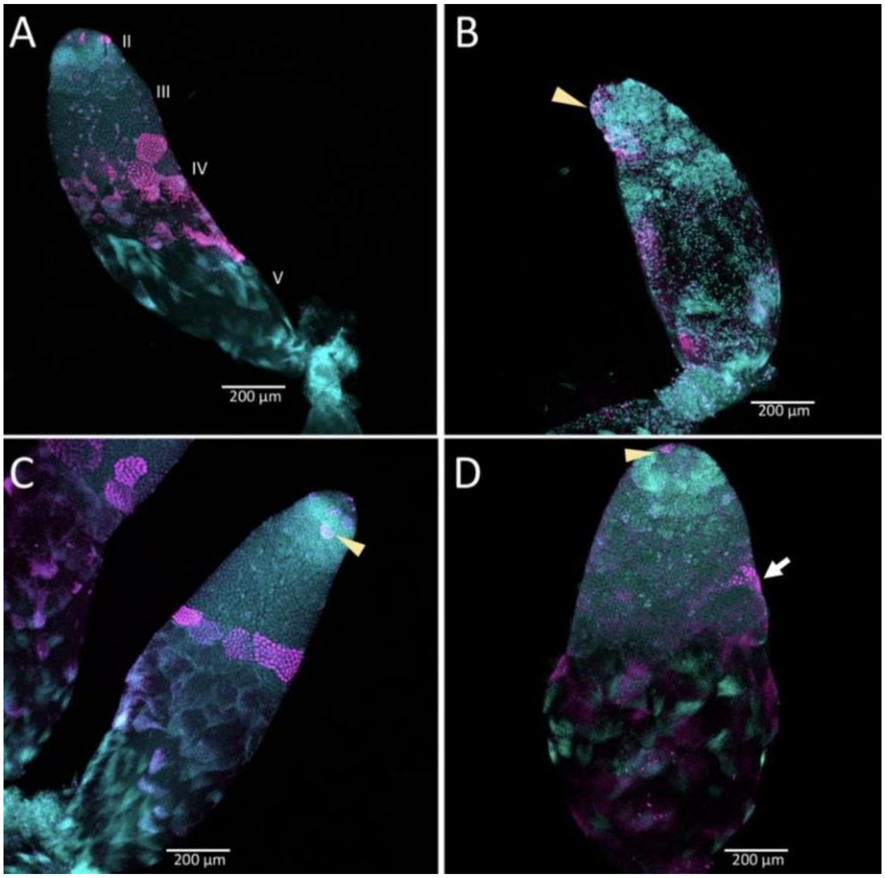

Figure 2.6 Timing of treatment during development determined the effect on testis structure of adults that develop from Dnmt1 (Panels B and D) knockdown males. In control testis tubules from males injected with ds-RED (A and C), spermatogonia divided mitotically in region II to form spermatocysts as is observed in untreated males. Mitotic spermatogonia are labeled with anti-phosophohistone 3 antibodies in region II of the testis tubule (C, arrowhead). Meiotic divisions also occured as in untreated males in ds-RED injected males. In our ds-RED control samples, a band of synchronously dividing spermatocysts, identified by positive anti-phosphohistone H3 antibody staining, was present at the interface between primary and secondary spermatocytes (A, C). In testes from males with Dnmt1 knockdown in the 3rd instar (B), the anterior tip of the testis tubule showed evidence of mitotic activity (arrowhead). However, there were fewer spermatocytes present and the spermatocysts in this region were disorganized and there was little evidence of the band of positive anti-phosphohistone H3 stained meiotic spermatocytes. The testis tubule structure from males treated with ds-Dnmt1 following meiosis at the 5th instar stage of development (D) was more similar to the controls than those treated at the 3rd instar stage of development. In ds-Dnmt1 males treated at the 5th instar stage, there were positive anti-phosphohistone H3 stained spermatogonia (arrowhead) and spermatocytes (arrow). There were differences between males treated as 5th instars with ds-RED males and ds-Dnmt1, however, including spermatocysts containing cells with highly condensed nuclei that were not present in the controls and fewer spermatocysts containing spermatids or spermatozoa. All images taken at 10X magnification.

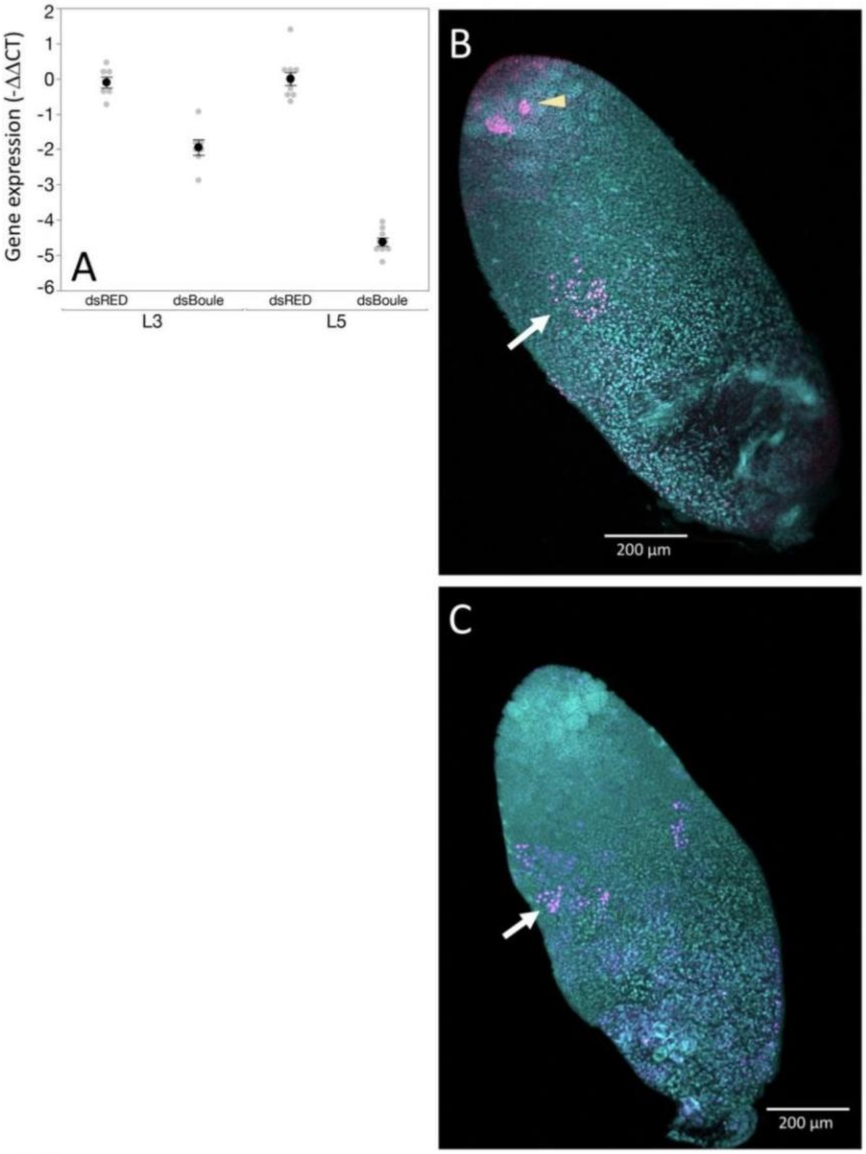

Figure 2.7
Timing of treatment during development determined the effect on testis structure of adults that develop from Dnmt1 (Panels B and D) knockdown males. In control testis tubules from males injected with ds-RED (A and C), spermatogonia divided mitotically in region II to form spermatocysts as is observed in untreated males. Mitotic spermatogonia are labeled with anti-phosophohistone 3 antibodies in region II of the testis tubule (C, arrowhead). Meiotic divisions also occured as in untreated males in ds-RED injected males. In our ds-RED control samples, a band of synchronously dividing spermatocysts, identified by positive anti-phosphohistone H3 antibody staining, was present at the interface between primary and secondary spermatocytes (A, C). In testes from males with Dnmt1 knockdown in the 3rd instar (B), the anterior tip of the testis tubule showed evidence of mitotic activity (arrowhead). However, there were fewer spermatocytes present and the spermatocysts in this region were disorganized and there was little evidence of the band of positive anti-phosphohistone H3 stained meiotic spermatocytes. The testis tubule structure from males treated with ds-Dnmt1 following meiosis at the 5th instar stage of development (D) was more similar to the controls than those treated at the 3rd instar stage of

development. In ds-Dnmt1 males treated at the 5th instar stage, there were positive anti-phosphohistone H3 stained spermatogonia (arrowhead) and spermatocytes (arrow). There were differences between males treated as 5th instars with ds-RED males and ds-Dnmt1, however, including spermatocysts containing cells with highly condensed nuclei that were not present in the controls and fewer spermatocysts containing spermatids or spermatozoa. All images taken at 10X magnification

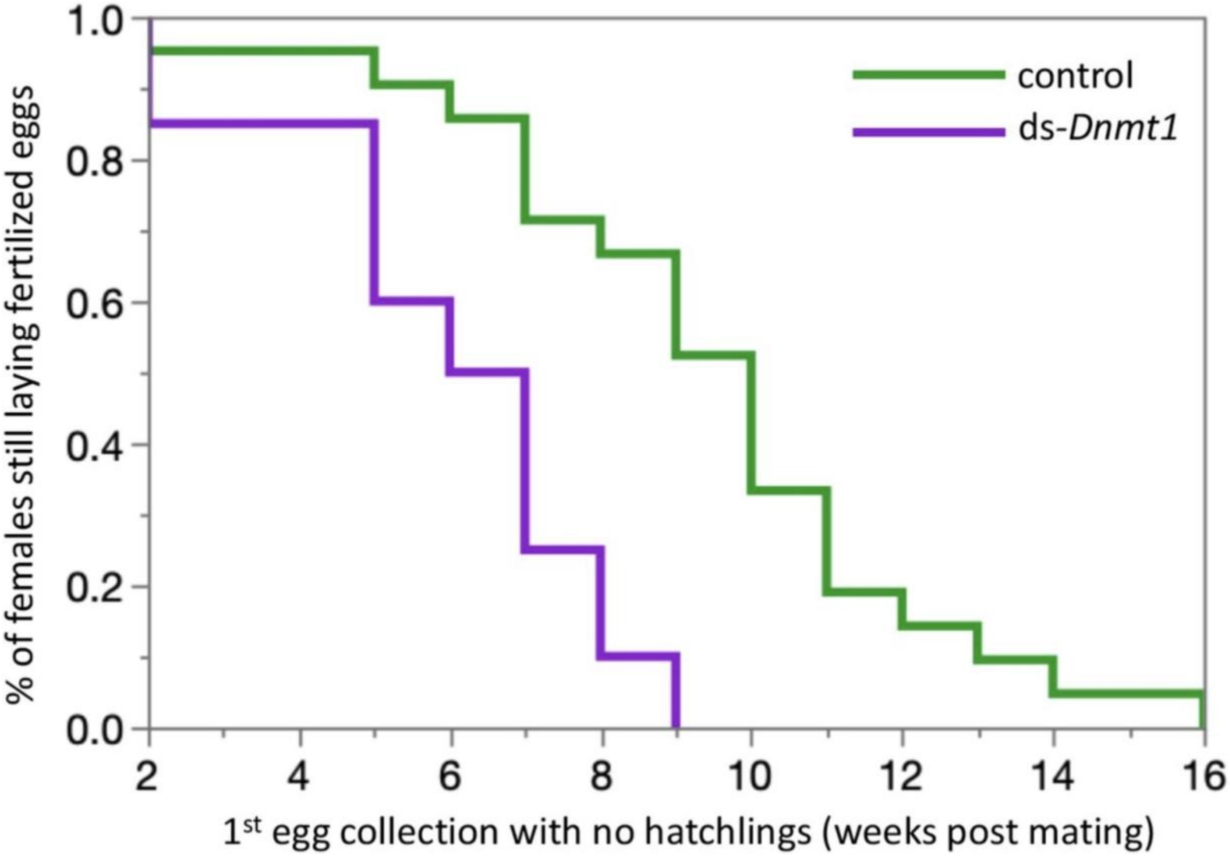

Figure 2.8 Females mated to control males lay fertilized eggs longer than females mated to Dnmt1 knockdown males. Eggs were collected twice per week and eggs allowed to develop to hatching. Eggs that did not hatch showed no sign of development, indicating that they had not been fertilized. Both treatments demonstrate a decrease in proportion of eggs that hatch over time, but the ds-Dnmt1 treatment group shows a faster decrease.

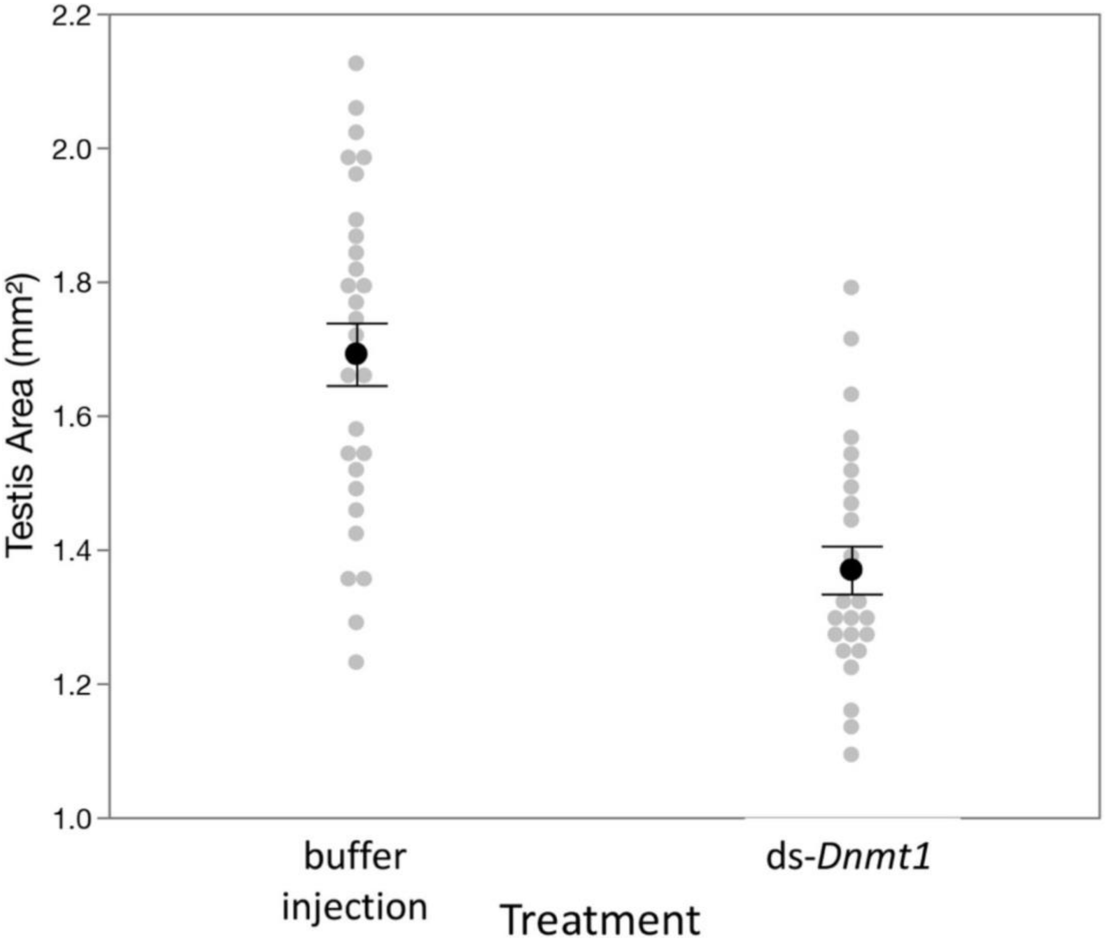

Figure 2.9 Downregulation of Dnmt1 in sexually mature males resulted in a loss of testis area following multiple matings. Control males allowed to mate with multiple females across three weeks had larger testes than males treated with ds-Dnmt1 at sexual maturity. Black dots and bars represent mean and SE. Grey dots represent data points for each individual tested.

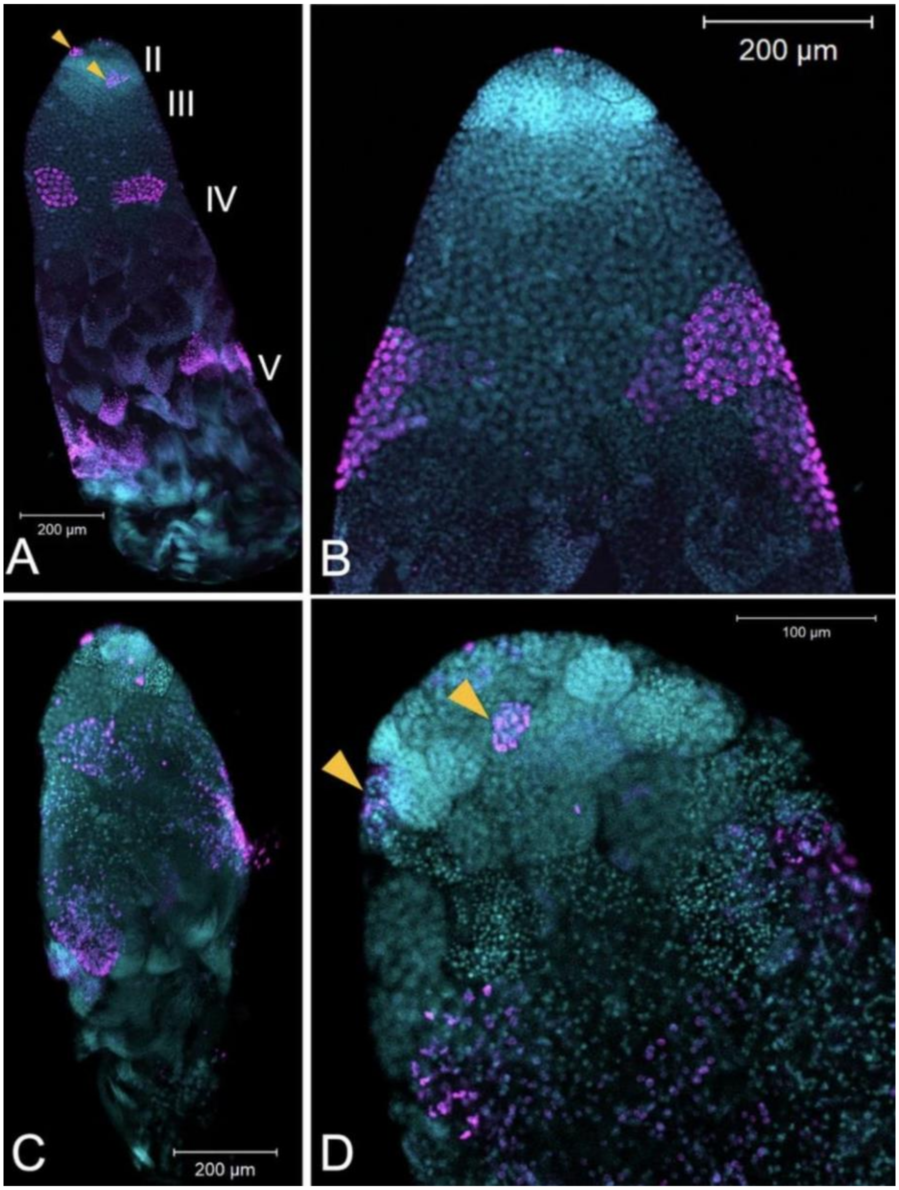

Figure 2.10 Testis structure breaks down in Dnmt1 knockdown males treated as adults after having sperm replenishment induced by mating activity. The regions of spermatogenesis were apparent in mated males following 3 weeks of mating activity in control testis tubules (A and B) and the evidence of both mitotic division in spermatogonia (B, arrowhead) and the band of meiotic divisions was clear from anti-phosphohistone H3 staining. In Dnmt1 knockdown males at low magnification (C), the anterior tip of the testis tubule looked relatively normal. However, the region containing both the primary and secondary spermatocytes was disorganized. Spermatocyst structure was broken down, and the nuclei of the primary and secondary spermatocytes had lost their characteristic structure (Ewen-Campen et al. 2013). Finally, there were fewer mature spermatids in the posterior end of the testis tubule. At higher magnification (D), it was apparent that nuclear structure in the anterior tip was also affected by the knockdown, both in the spermatogonia and spermatocytes. Spermatogonia nuclei in the Dnmt1 knockdown testis tubules (D) were more condensed than in the control testis tubules (B), although they still seemed to be organized into spermatocysts. Spermatocyte nuclei, however, were fewer in

number than in controls, did not have their characteristic shape (Ewen-Campen et al. 2013, Economopoulos & Gordon 1971), and were not organized in spermatocytes. A and C:10X magnification, B and D: 20X magnification.