The essential role of *Dnmt1* in gametogenesis in the large milkweed bug *Oncopeltus fasciatus*

Joshua T. Washington[1], Katelyn R. Cavender[1], Ashley U. Amukamara[1], Elizabeth C. McKinney[1], Robert J. Schmitz[2], Patricia J. Moore[1,*]

[1] Department of Entomology, University of Georgia

[2] Department of Genetics, University of Georgia

*Patricia J. Moore, 413 Biological Sciences Building, Department of Entomology, University of Georgia, Athens, GA, 30602; (706) 542-0169

Email: pjmoore@uga.edu

Patricia J. Moore: https://orcid.org/0000-0001-9802-7217

Robert J. Schmitz: https://orcid.org/0000-0001-7538-6663

Keywords

DNA methylation, epigenetics, *Dnmt1*, spermatogenesis, germ cells, *Oncopeltus fasciatu*

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. We show that the maintenance DNA methyltransferase, DNMT1, affects meiosis and is essential to fertility in milkweed bugs, *Oncopeltus fasciatus*, while DNA methylation is not required in somatic cells. Our 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. Our 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.

Introduction

Despite the apparent ubiquity of DNA methylation across the eukaryotic tree of life (Schmitz et al. 2019, Lewis et al. 2020), in the insects there is considerable 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*, 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 *Dnmt1* is required for the development of sperm both during 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 development reflected its role in maintenance of DNA.

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. Contrary to this expectation, *Dnmt1* expression was highest during larval development, a time during which cells are dividing, and expression went down in the adult stages, where there is relatively less cell division occurring (Figure 1A; ANOVA, $F = 170.768$, d.f. = 5, 54, $p < 0.001$). This

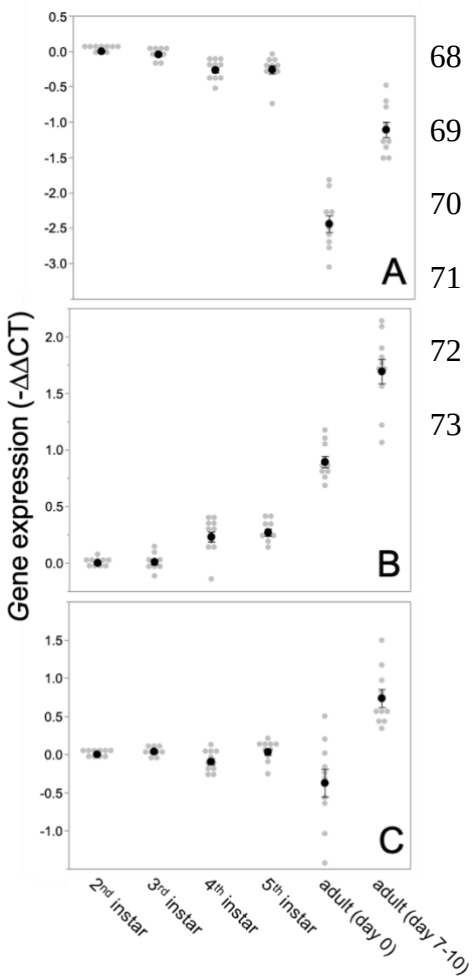

Figure 1. Expression levels of *Dnmt1* do not mirror those of genes known to function in germ cell identity and development. (A) Expression levels of *Dnmt1* did not change across nymphal development but did vary during adult sexual maturation. *Dnmt1* expression went down when males emerged as adults, although it recovered with sexual maturity. (B) *Boule* increased across development. Expression was lowest during the 2nd and 3rd instar stages before meiosis is initiated. *Boule* expression increased in the 4th and 5th instar stages where meiosis is initiated. Finally, *Boule* expression increased in adult stages and was highest in the testis of sexually mature adults. (B) *Vasa* was expressed across all stages of nymphal development. Expression was low in newly emerged adults but increased significantly during sexual maturation. Black dots and bars represent mean and SE. Grey dots represent data points for each sample tested. Gene expression was standardized per individual.

expression pattern differed from what we observed for *Boule*, a gene with a well-characterized role in meiosis (Figure 1B; ANOVA, $F = 145.799$, d.f. = 5, 54, $p < 0.001$). *Dnmt1* expression pattern also differed from *Vasa*, a highly conserved marker of the germline (Figure 1C; ANOVA, $F = 16.008$, d.f. = 5, 54, $p < 0.001$).

Given that *Dnmt1* is required during cell division to restore methylation patterns, we predicted that expression patterns would be similar between the sexes during development. If *Dnmt1* has an additional function during gametogenesis, however, we would predict that there would be differences in expression patterns that reflect differences in the pattern of gametogenesis between males and females. Expression of *Dnmt1* in whole body samples was lower in sexually mature adult males than sexually mature adult females, but male and female nymphs had the same expression levels (Supplementary materials, Figure S1). Thus, expression patterns indicated a similar function during larval development, when cell division is occurring in both sexes, but also suggested differences between patterns of gametogenesis between males and females as adults.

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 control and ds-*Dnmt1* injected males (Figure 2). 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

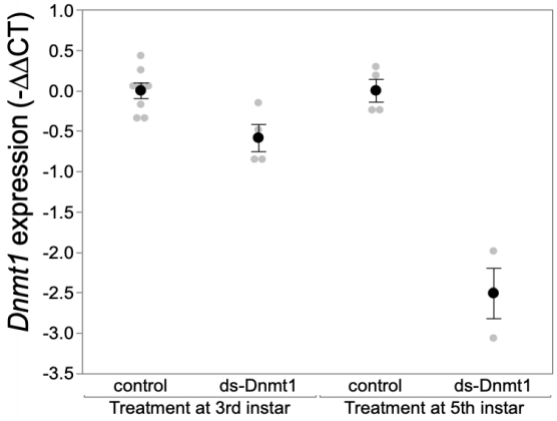

Figure 2. Expression of *Dnmt1* was reduced in the testes of adult males treated with ds-*Dnmt1* at both stages of development. Relative gene expression is standardized to expression levels in control treatments. Black dots and bars represent mean and SE. Grey dots represent data points for each individual tested.

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 (Supplementary materials, Figure S2). 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 at which meiosis occurs, the 5th instar stage, had no effect on testis size (Figure 3). In our overall 2-way ANOVA there were statistically significant effects of stage of development at injection ($F = 142.326$, d.f. = 1, $p < 0.001$), treatment ($F = 18.568$, d.f. = 1, $p < 0.001$), and a statistically significant interaction between stage of development at injection and treatment ($F = 39.947$, d.f. = 1, $p < 0.001$). Stage of development at injection had an effect on testis area, even

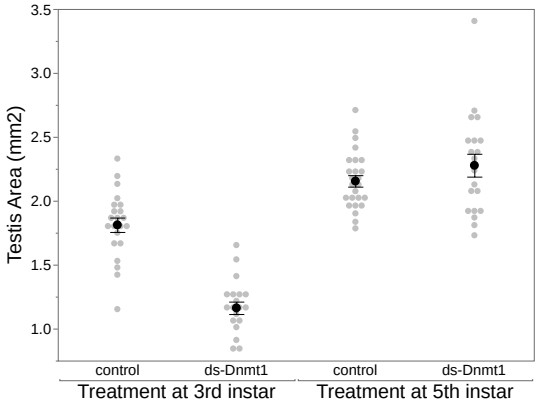

Figure 3. Early stage injection and ds-*Dnmt1* both affected testis size in males developing from treated males. The testes of control injected males treated at the 3rd instar stage of development are smaller than control males treated at the 5th instar stage of development, likely a result of injection injury. Downregulation of *Dnmt1* during the 3rd instar stage of development significantly reduced the size of the testis in sexually mature males compared to control males treated at the 3rd instar stage of development. There was no effect of downregulation of *Dnmt1* on testis size when treatment occurred at 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.

in control injections. Injecting 3rd instar nymphs reduced adult testis area, suggesting an injury effect independent of treatment. However, ds-*Dnmt1* males had highly statistically significantly smaller testes than control males at sexual maturation when treated at the 3rd instar stage ($F = 74.155$, d.f. = 1, 39, $p < 0.001$, indicating a treatment-specific reduction in testis size in addition to the injection effect in the 3rd instar. Males at the 5th instar stage showed no statistically significant difference in testis area at sexual maturation between treatments ($F = 1.698$, d.f. = 1, 43, $p = 0.199$).

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 4). Control males injected in the 3rd or 5th instar stage of development showed the expected structure of the testis tubule (Figure 4A 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 4C, 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 (Supplementary materials, Figure S3).

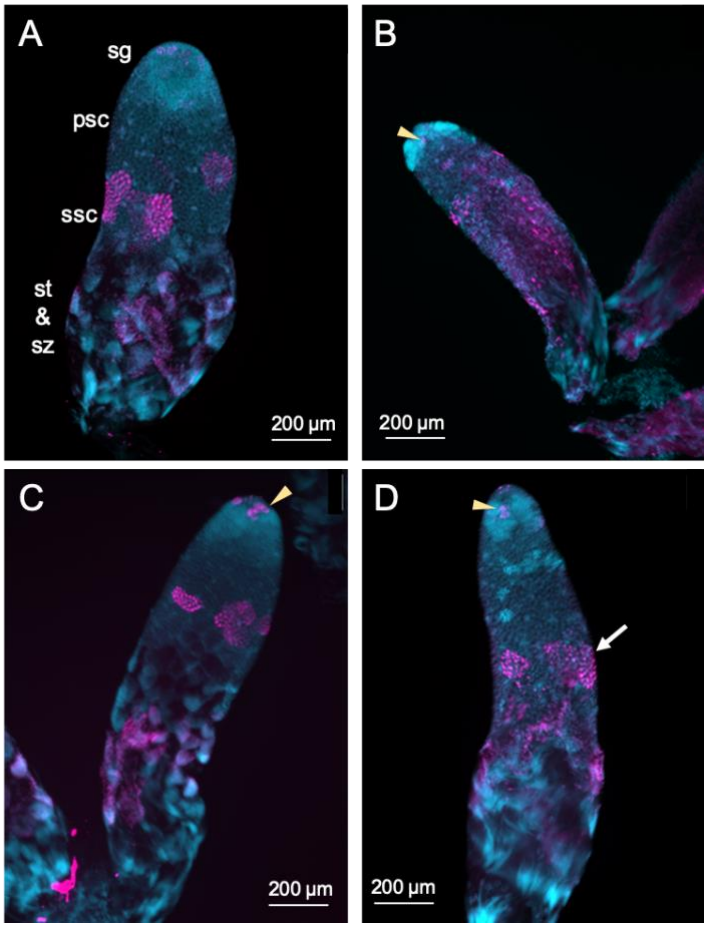

Figure 4. Timing of treatment during development determined the effect on testis structure of adults that develop from *Dnmt1* (Panels C and D) knockdown males. In control testis tubules (A and B), spermatogenesis progresses from the tip of the testis tubule. The germline stem cell and spermatogonia (sg) are located at the anterior end of the tubule. Spermatogonia divide mitotically to form spermatocysts containing 64 spermatogonia (Economopoulos & Gordon 1971, Ewen-Campen et al. 2013). Mitotic spermatogonia are labeled with anti-phosophohistone 3 antibodies in this region of the testis tubule (C arrowhead). *Oncopeltus fasciatus* undergoes inverted meiosis (Viera et al. 2009), primary spermatocytes (psc) undergo the first meiotic division to produce diploid secondary spermatocytes (ssc). The meiotic division of the ssc produce the haploid spermatids (st) and spermatozoa (sz). In our 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 looked relatively normal and there was 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 control males and ds-*Dnmt1* treated males, however, including spermatocysts containing cells with highly condensed nuclei that were not present in the controls. All images taken at 10X magnification.

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 4B). 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, 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 4D). Mitotic activity was apparent in the spermatogonia and most testis tubules had evidence of mature sperm and α-pHH3 stained spermatocysts at the junction between primary and secondary spermatocytes. 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. 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 5; 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, $p < 0.001$; Supplementary materials, Figure S4). The

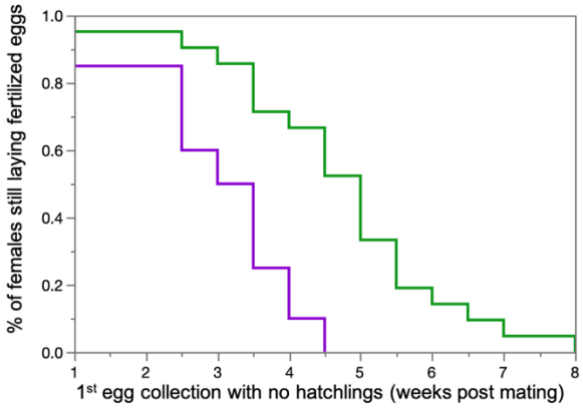

Figure 5. Females mated to control males lay fertilized eggs longer than females mated to *Dnmt1* knockdown males. Eggs were collected twice per week and eggs allowed to develop to hatching. Eggs that did not hatch showed no sign of development, indicating that they had not been fertilized. Both treatments demonstrate a decrease in proportion of eggs that hatch over time, but the ds-*Dnmt1* treatment group shows a faster decrease.

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 6). 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 6A 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

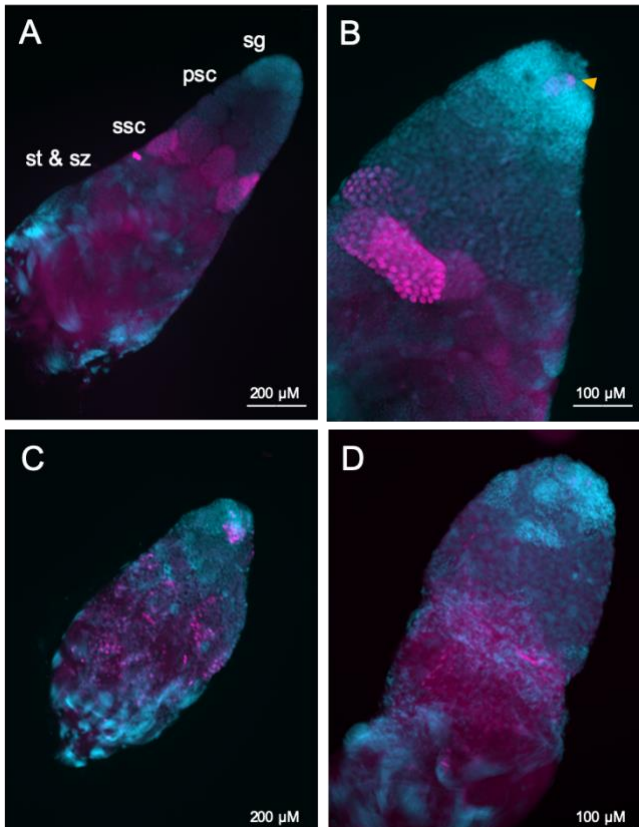

Figure 6. Testis structure breaks down in *Dnmt1* knockdown males treated as adults after having sperm replenishment induced by mating activity. The regions of spermatogenesis were apparent in mated males following 3 weeks of mating activity in control testis tubules (A and B) and the evidence of both 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.

197 than in the testis tubules of control males (Figure 6C and D). The most noticable effect was seen
198 in the region containing the spermatocytes. There were few primary spermatocytes in the testis
199 tubule and α-pHH3 positively stained spermatocysts were rarely observed, indicating few
200 spermatocysts undergoing meiosis.

201 **Discussion**

202 The function of DNA methylation and the DNA methylation enzymes across the insect tree of
203 life has been widely debated, particularly in light of the extreme variation in presence of this
204 chromatin modification and the diversification of the enzymatic toolkit required to methylate
205 DNA *de novo* and maintain DNA methylation patterns (Bewick et al. 2016, Lyko 2018, Lewis et
206 al. 2020). This evolutionary pattern is particularly intriguing given that in a number of insect
207 groups the maintenance methyltransferase DNMT1 is essential to gametogenesis, and thus is
208 tightly tied to fitness, even in species where DNA methylation itself is absent. Here we showed
209 that *Dnmt1* expression affects meiosis without any obvious phenotypic effects in somatic tissue.
210 The effect of downregulating *Dnmt1* in males, as in females (Amukamara et al. 2020), was
211 specific to the germ cells. Nymphs treated as early as the 3rd instar stage develop normally and
212 are indistinguishable from control adults. The downregulation of *Dnmt1* did result in reduction of
213 DNA methylation but there were no morphological impacts to the RNAi-treated individuals. Our
214 results show that the function of *Dnmt1* in germ cells is conserved across the sexes within a
215 species, and other results document that *Dnmt1* expression is required for oogenesis among
216 species. This raises a conundrum when we look across the insect tree of life. How can a gene
217 required for such a fundamental fitness activity in examples from such a diverse group of insects
218 also be so evolutionarily labile? This suggests that the function *Dnmt1* in germ cell development
219 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. 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, as was observed in female *O. fasciatus* (Amukamara et al. 2020). 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*. However, we do not think that the function of *Dnmt1* in spermatogenesis is unique to *O. fasciatus*. 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.

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

289 (Amukamara et al. 2020), greater than a two-fold reduction in the percent methylated CpG
290 residues. In *Dnmt1* knockdown females, there was a compete loss of oocyte production when
291 DNA methylation was reduced to this level while in males the phenotype of the 5th instar *Dnmt1*
292 knockdowns was close to normal. This disconnect between reduction of DNA methylation and
293 phenotypic effects mirrors what has been seen in the beetle, *T. castaneum* (Schulz et al. 2018). In
294 this species, the reduction of DNA methylation is an evolved difference rather than an
295 experimental effect, but the results are similar to what we have observed in *O. fasciatus*; DNA
296 methylation is not required for function of somatic cells, but downregulation of *Dnmt1*
297 expression leads to specific germ cell effects. The lack of correlation between extent of
298 methylation and function in germ cells strengthens our hypothesis that there may be a pleiotropic
299 function for *Dnmt1* in germ cells that acts independently of DNA methylation levels. Also
300 consistent with this idea is the finding that *Dnmt1* expression is elevated in both the ovaries and
301 testes relative to somatic tissue in the migratory locust, *Locusta migratoria* (Robinson et al.
302 2016). Alternatively, DNA methylation may have a specific role in the germ cells that it does not
303 play in somatic cells. Perhaps DNA methylation is required for proper gene expression in germ
304 cells or it may be required for transposon silencing only in germ cells. Another possibility is that
305 DNA methylation state may be a prerequisite for successful completion of meiosis and not
306 mitosis. For example, *O. fasciatus* has holocentric chromosomes and undergoes inverted meiosis
307 (Viera et al. 2009). During mitosis chromosomes have a kinetocore that extends across the
308 majority of the chromosome but in meiosis, the kinetochore plate is missing and microtubules
309 extend into the chromosome (Comings & Okada 1972). One could imagine that DNA
310 methylation might be required for this interaction. However, the complete lack of methylation
311 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.

Conclusion

Dnmt1 expression is required for germ cell development in both male and female *O. fasciatus*. The block to germ cell development in *Dnmt1* knockdowns appeared to be associated with meiosis, although it was not a simple block to progression through meiosis as germ cells are lost from the testes. Thus, *Dnmt1* may be required for germ cell viability. It still remains to be determined if the block in gametogenesis depends on DNA methylation or an alternative function of *Dnmt1*, as suggested by the requirement for *Dnmt1* during gametogenesis in an insect with a non-methylated genome (Schultz et al. 2018). Whatever the specific function of *Dnmt1* in gametogenesis, the requirement for this enzyme in such a critical fitness function as the production of gametes in representatives of different groups of insects raises important questions as to how this enzyme and the entire methylation toolkit has evolved across the insect tree of life. Functional analysis of *Dnmt1* in the insect groups where it is found, and study of how these functions are replaced in the species where it is no longer found, will be essential for understanding the evolution of this important base modification.

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 development in males. Groups of nymphs were staged and flash frozen in liquid nitrogen and stored at -80° C. Males were able to be distinguished from females reliably in the 4th and 5th instar stages, so at these stages the nymphs were separated by sex. We also collected virgin males on the day of adult emergence and after sexual maturation at 7 days post-adult-emergence. We collected and tested these samples at the same time as the data reported for females (Amukamara et al. 2020). Because 2nd and 3rd instar nymphs cannot be reliably sexed, the data presented here is the same as that presented in the previous publication. Further, we used the female data to examine sex differences in expression. The female data has been deposited in the DRYAD Digital Repository (https://doi.org/10.5061/dryad.kwh70rz0m).

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 reactions. 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 ΔΔCT 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 Pro v14. 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 NaH_2PO_4).

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 injections at either the 3rd

379 instar or 5th instar stage of development. Previous studies from our lab have shown no difference
380 in buffer alone controls or non-specific ds-RNA (Bewick et al. 2019). For further information on
381 controls and testing for potential off-target effects please see Amukamara et al. 2020. Nymphs
382 were anaesthetized at 4° C for 20 minutes prior to injection. Nymphs were injected in the
383 abdomen using pulled glass capillary needles (Sutter Instrument Company micropipette puller
384 model P-97, Novato, CA) between the third and fourth abdominal segments (Chesebro et al.
385 2009). Nymphs were injected with 2 µL volume for all injections. Following injections, nymphs
386 were placed in individual petri dishes and monitored for development. Date of adult emergence
387 was recorded. We did not do a power analysis, but based on preliminary data on the strength of
388 the effect we aimed for 25 individual males for each treatment. Males were randomly assigned to
389 a treatment group. Not all males survived to age of dissection, resulting in final sample sizes of
390 22 and 19 for 3rd instar males injected with buffer and ds-*Dnmt1*, respectively and 25 and 20 for
391 5th instar males injected with buffer and ds-*Dnmt1*, respectively.

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

398 *Testis tubule structure*: *O. fasciatus* testes contain 7 individual testis tubules surrounded by a
399 relatively impermeable, autofluorescent membrane. Individual testis tubules were removed from
400 the outer membranous sheath for fixation and staining. Males from each treatment were dissected
401 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 (0.5 μg/mL PBT) to visualize nucleic acids. Stained tubules were mounted in Mowiol 4-88 mounting medium (Sigma-Aldrich, St. Louis, MO) and visualized with 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 7- to 10-day old males from each treatment at both developmental stages (3rd and 5th instar) using a Qiagen Allprep DNA/RNA Mini Kit (Qiagen, Venlo, The Netherlands). Expression levels for *Dnmt1* was analyzed using qRT-PCR as described above. Given our extensive experience with our RNAi being successful in knocking down expression of *Dnmt1*, we did not test a sample size adequate for statistical analysis, but the pattern across a minimum number of samples (3 to 7 individuals from any treatment) showed consistent effect on expression in our ds-*Dnmt1* injected males.

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 buffer control injections using a pulled glass capillary needle between the third and fourth abdominal segments (Chesebro et al. 2009). 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-

447 day-old virgin female was placed with the male with fresh cotton wool. The 3rd female and
448 experimental male were given one week to mate before the male was removed from the petri
449 dish for analysis of testis size and structure. The female was maintained for her lifetime,
450 provided with *ad libitum* food and water. The eggs produced by the 3rd female were collected
451 from the petri dish twice a week at 3-4 day intervals and the oviposition substrate replaced with
452 fresh cotton wool. The eggs collected were placed in a separate container and allowed to develop
453 to hatching (approximately 7-10 days following collection) and then frozen at -20° C until
454 assayed. We recorded the first collection date for which no eggs hatched. We analyzed the time
455 to end of fertilized eggs due to sperm depletion relative to treatment using a survival analysis
456 (Wilcoxon Rank Sum test) using JMP Pro v14.1. Not all males survived to date of dissection, or
457 their mates died during the course of egg collection, requiring these males to be removed from
458 the analysis, resulting in a sample size of 21 control and 20 ds-*Dnmt1* treated males in the final
459 fecundity analysis.

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

464 *Testis tubule structure*: Testis tubules from mated males were isolated, fixed and stained as
465 described above.

466 **Author Contributions**

467 PJM and RJS contributed conception and design of the study. JW, KC, AUA, LG, ECM, and
468 PJM collected the data. All authors contributed to the analysis of the data. JW and PJM wrote the
469 first draft of the manuscript, with contributions from KC. All authors contributed to manuscript
470 revision and all authors read and approved the submitted version.

471 **Acknowledgments**

The authors acknowledge Allen J. Moore and other members of the Moore lab for many helpful discussions. We acknowledge Luvika Gupta, who's undergraduate research provided preliminary data used in our experimental design. We also would like to acknowledge Tyler Earp for preparing and analyzing low throughput whole genome bisulfite sequencing data.

Competing Interests

The authors have no competing interests in regards to this work.

References

Amukamara A.U., J. T. Washington, Z. Sanchez, E.C. McKinney, A. J. Moore, R. J. Schmitz, P. J. Moore. More than DNA methylation: does pleiotropy drive the complex pattern of evolution of Dnmt1? *Frontiers in Ecology and Evolution* 8, 4 (2020).

Bewick, A.J., K. J. Vogel, A. J. Moore, R. J. Schmitz. Evolution of DNA methylation across insects. *Molecular Biology and Evolution* 34, 654-66 (2016).

Bewick, A.J., Z. Sanchez, E. C. Mckinney, A. J. Moore, P. J. Moore, R. J. Schmitz. *Dnmt1* is essential for egg production and embryo viability in the large milkweed bug, *Oncopeltus fasciatus*. *Epigenetics & Chromatin* 12, 6 (2019).

Chesebro, J., S. Hrycaj, N. Mahfooz, A. Popadić. Diverging functions of Scr between embryonic and post-embryonic development in a hemimetabolous insect, *Oncopeltus fasciatus*. *Developmental Biology* 329, 142-151 (2009).

Comings, D.E., T. A. Okada. Holocentric chromosomes in *Oncopeltus*: kinetochore plates are present in mitosis but absent in meiosis. *Chromosoma* 37, 177-192 (1972). https://doi.org/10.1007/BF00284937

Cunningham, C.B., L. Ji, R. A. W. Wiberg, J. Shelton, E. C. McKinney, D. J. Parker, R. B. Meagher, K. M. Benowitz, E. M. Roy-Zokan, M. G. Ritchie, S. J. Brown, R. J. Schmitz, A. J. Moore. The genome and methylome of a beetle with complex social behavior, *Nicrophorus vespilloides* (Coleoptera: Silphidae). *Genome Biology and Evolution* 7, 3383-3396 (2015).

Eberhart, C.G., J. Z. Maines, S. A. Wasserman. Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. *Nature* 381, 783-785 (1996).

Economopoulos, A.P. H. T. Gordon. Growth and differentiation of the testes in the large milkweed bug, *Oncopeltus fasciatus* (Dallas). *Journal of Experimental Zoology* 177, 391-405 (1971).

Ewen-Campen, B., T. E. Jones, C. G. Extavour. Evidence against a germ plasm in the milkweed bug *Oncopeltus fasciatus*, a hemimetabolous insect. *Biology Open* 2, 556-568 (2013).

Gegner, J., T.Gegner, H. Vogel, A. Vilcinskas. Silencing of the DNA methyltransferase 1 associated protein 1 (DMAP1) gene in the invasive ladybird *Harmonia axyridis* implies a role of the DNA methyltransferase 1-DMAP1 complex in female fecundity. *Insect Molecular Biology* 29, 148-159 (2020). https://doi.org/10.1111/imb.12616

Glastad, K.M., B. G. Hunt, M. A. Goodisman. Epigenetics in insects: genome regulation and the generation of phenotypic diversity. *Annual Review of Entomology* 64, 185-203 (2019). doi.org/10.1146/annurev-ento-011118-111914

Hans, F., S. Dimitrov. Histone H3 phosphorylation and cell division. *Oncogene* 20, 3021-3027 (2001).

Lewis S.H., L. Ross, S. A. Bain, E. Pahita, S. A. Smith, R. Cordaux, et al. Widespread conservation and lineage-specific diversification of genome-wide DNA methylation patterns across arthropods. *PLoS Genetics* 16, e1008864 (2020). https://doi.org/10.1371/journal.pgen.1008864

Livak, K.J., T. D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods* 25, 402-408 (2001).

Lyko, F. The DNA methyltransferase family: a versatile toolkit for epigenetic regulation. *Nature Reviews Genetics* 19, 81-92 (2018).

Medrano, J.V., C. Ramathal, H. N. Nguyen, C. Simon, R. A. Reijo Pera. Divergent RNA-binding proteins, DAZL and VASA, induce meiotic progression in human germ cells derived in vitro. *Stem cells* 30, 441-451 (2012).

Meinzer, F., S. Dobler, A. Donath, J. N. Lohr. Robust reference gene design and validation for expression studies in the large milkweed bug, *Oncopeltus fasciatus*, upon cardiac glycoside stress. *Gene* 710, 66-75 (2019).

Prigent, C. S. Dimitrov. Phosphorylation of serine 10 in histone H3, what for?. *Journal of Cell Science* 116, 3677-3685 (2003).

Robinson, K.L., D. Tohidi-Esfahani, F. Ponton, S. J. Simpson, G. A. Sword, N. Lo. Alternative migratory locust phenotypes are associated with differences in the expression of genes encoding the methylation machinery. *Insect Molecular Biology* 25, 105-115 (2016).

Schmidt, E. D., E. Sehn, A. Dorn. Differentiation and ultrastructure of the spermatogonial cyst cells in the milkweed bug, *Oncopeltus fasciatus*. *Invertebrate Reproduction & Development* 42, 163-178 (2002). DOI:10.1080/07924259.2002.9652773

Schmitz, R. J., Z. A. Lewis, M. G. Goll. DNA methylation: shared and divergent features across eukaryotes. *Trends in Genetics* 35, 818-827 (2019). doi.org/10.1016/j.tig.2019.07.007

Schmitz, R.J., Y. He, O. Valdés-López, S. M. Khan, T. Joshi, M. A. Urich, J. R. Nery, B. Diers, D. Xu, G. Stacey, J. R. Ecker. Epigenome-wide inheritance of cytosine methylation variants in a recombinant inbred population. *Genome Research* 23, 1663-1674 (2013).

Schultz, M.D., R. J. Schmitz, J. R. Ecker. 'Leveling' the playing field for analyses of single-base resolution DNA methylomes. *Trends in Genetics* 28, 583-585 (2012). DOI:https://doi.org/10.1016/j.tig.2012.10.012

Schulz, N.K., C. I. Wagner, J. Ebeling, G. Raddatz, M. F. Diddens-de Buhr, F. Lyko, J. Kurtz. Dnmt1 has an essential function despite the absence of CpG DNA methylation in the red flour beetle *Tribolium castaneum*. *Scientific Reports* 8, 1-10 (2018). https://doi.org/10.1038/s41598-018-34701-3

Shah, C., M. J. VanGompel, V. Naeem, Y. Chen, T. Lee, N. Angeloni, Y. Wang, Y. E. Y. Xu. Widespread presence of human BOULE homologs among animals and conservation of their ancient reproductive function. *PLoS Genetics* 6, e1001022 (2010). doi.org/10.1371/journal.pgen.1001022

Tanaka, S.S., Y. Toyooka, R. Akasu, Y. Katoh-Fukui, Y. Nakahara, R. Suzuki, M. Yokoyama, T. Noce. The mouse homolog of Drosophila Vasa is required for the development of male germ cells. *Genes & Development* 14, 841-853 (2000).

560 Ulrich, M. S., J. R. Nery, R. Lister, R. J. Schmitz, J. R. Ecker. MethylC-seq library
561 preparation for base-resolution whole-genome bisulfite sequencing. *Nature Protocols*
562 10, 475–483 (2015). doi: 10.1038/nprot.2014.114

563 Viera, A., J. Page, J. Rufas. Inverted meiosis: the true bugs as a model to study. In:
564 Benavente, R., Volff J.-N. (eds): Meiosis. Genome Dynamics, Basel, Karger, 2009, vol
565 5, pp 137-156 (2009). https://doi.org/10.1159/000166639
566 Yajima, M., G. M. Wessel. The multiple hats of Vasa: its functions in the germline and in
567 cell cycle progression. *Molecular reproduction and development* 78, 861-867 (2011)

568 Zemach, A., I. E. McDaniel, P. Silva, D. Zilberman. Genome-wide evolutionary analysis of
569 eukaryotic DNA methylation. *Science* 328, 916-919 (2010).