The relative importance of DNA methylation and Dnmt2-mediated epigenetic regulation on *Wolbachia* densities and cytoplasmic incompatibility

Wolbachia pipientis is a worldwide bacterial parasite of arthropods that infects host germline cells and manipulates host reproduction to increase the ratio of infected females, the transmitting sex of the bacteria. The most common reproductive manipulation, cytoplasmic incompatibility (CI), is expressed as embryonic death in crosses between infected males and uninfected females. Specifically, *Wolbachia* modify developing sperm in the testes by unknown means to cause a post-fertilization disruption of the sperm chromatin that incapacitates the first mitosis of the embryo. As these *Wolbachia*-induced changes are stable, reversible, and affect the host cell cycle machinery including DNA replication and chromosome segregation, we hypothesized that the host methylation pathway is targeted for modulation during cytoplasmic incompatibility because it accounts for all of these traits. Here we show that infection of the testes is associated with a 55% increase of host DNA methylation in *Drosophila melanogaster*, but methylation of the paternal genome does not correlate with penetrance of CI. Overexpression and knock out of the *Drosophila* DNA methyltransferase Dnmt2 neither induces nor increases cytoplasmic incompatibility. Instead, overexpression decreases *Wolbachia* titers in host testes by approximately 17%, leading to a similar reduction in CI levels. Finally, strength of CI induced by several different strains of *Wolbachia* does not correlate with levels of DNA methylation in the host testes. We conclude that DNA methylation mediated by *Drosophila*'s only known methyltransferase is not required for the transgenerational sperm modification that causes CI.

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8   Introduction

9   *Wolbachia pipientis*, an obligate intracellular bacteria, is estimated to infect

10   approximately 40% of all arthropod species (Zug & Hammerstein, 2012). This widespread

11   prevalence can be attributed to efficient maternal transmission of the infection, intermediate rates

12   of horizontal transmission to new hosts, and strong manipulations of the host reproductive system

13   to enhance its maternal transmission (Stouthamer, Breeuwer & Hurst, 1999; Serbus et al., 2008).

14   These sexual alterations all act to increase the number of infected females within a population

15   and include male-killing, feminization, parthenogenesis, and cytoplasmic incompatibility (CI). CI

16   is the most common defect observed in *Wolbachia*-infected hosts and has been documented in

17   numerous species (Serbus et al., 2008).

18   CI acts as a post-fertilization mating barrier by preventing the development of embryos

19   from uninfected females that are mated with *Wolbachia*-infected males. This zygotic defect can

20   be rescued, however, by females infected with the same strain of *Wolbachia* present in the male.

21   This rescue capability gives a strong fitness advantage to *Wolbachia*-infected females and can

22   lead to rapid sweeps of the infection through host populations. For instance, CI-inducing

23   *Wolbachia* have been able to spread across most of the *Drosophila simulans* population in eastern

24   Australia in less than a decade (Kriesner et al., 2013). Cytoplasmic incompatibility is also a major

25   isolation barrier between young sibling species (Bordenstein, O'Hara & Werren, 2001; Jaenike et

26   al., 2006; Miller, Ehrman & Schneider, 2010) and is currently being used as a genetic drive

27   mechanism to eliminate dengue virus in *Aedes aegypti* populations (Moreira et al., 2009; Bian et

28   al., 2010; Walker et al., 2011) and to generally reduce mosquito population sizes (Laven, 1967;

29   O'Connor et al., 2012).

30   The evolutionary, ecological, and medical importance of cytoplasmic incompatibility has

31   fueled decades of research seeking to understand its underlying mechanisms. However, apart

32   from studies that suggest the host genes JhI-26 and HIRA are involved (Zheng et al., 2011; Liu et

33   al., 2014), it remains unknown how *Wolbachia* in the testes encode a sperm modification that

34   renders embryos inviable. Previous work elucidated a few post-fertilization hallmarks of CI, most

35   of which are associated with defects in the paternal genome during embryogenesis. These

36   changes include a failure of maternal histones to deposit correctly, prolonged or incomplete

37   replication of the paternal DNA, and failed condensation of the paternal chromosomes (Breeuwer

38   & Werren, 1990; Callaini, Dallai & Riparbelli, 1997; Landmann et al., 2009). The alterations of

39   the paternal chromatin and host cell cycle lead to a failure of the first mitosis followed by

40   embryonic death. Interestingly, *Wolbachia* are not actually present within the sperm of their

41   hosts, indicating a semi-permanent modification of the paternal genome that is

42   transgenerationally transmitted to the egg (Clark et al., 2008).

43   Several assumptions can be made about the paternal genome modification underlying

44   cytoplasmic incompatibility including:

45   (i)   It targets host pathways that are highly conserved across numerous host species
46   (ii)   It involves a semi-permanent but reversible alteration to the paternal genome
47   (iii)   It must be able to affect histone recruitment, DNA replication, and chromosome

48   condensation

49   Working under these assumptions, we selected the host DNA methylation pathway as a probable

50   target for *Wolbachia*. Methylation is a stable, yet reversible, modification to DNA that could be

51   sex-specific and easily rescued by infected females. It also has the capability to modulate many

52   cell cycle functions including chromosome condensation and histone recruitment (Bird, 2001;

53   Harris & Braig, 2003; Weber & Schübeler, 2007) and has previously been hypothesized to play a

54   role in CI (Negri, 2011; Saridaki et al., 2011; Ye et al., 2013b; Liu et al., 2014). While the role of

55   DNA methylation in insects is not fully understood it is a highly conserved pathway that shows

56   strong upregulation during embryogenesis (Field et al., 2004). Finally, the ability of bacteria to

57   alter host methylation and chromatin structure is increasingly being recognized (Gómez-Díaz et

58   al., 2012; Bierne, Hamon & Cossart, 2012) and previous work shows that *Wolbachia* infection in

59   particular alters the host methylation profile in both leafhoppers and mosquitoes (Negri et al.,

60   2009; Ye et al., 2013a).

61   Here we use the model organism *Drosophila melanogaster* infected with the *w*Mel strain

62   of *Wolbachia* to determine the role of host DNA methylation in cytoplasmic incompatibility. *D.*

63   *melanogaster* flies utilize just one canonical DNA methyltransferase, *Dnmt2* (Lyko, Ramsahoye

64   & Jaenisch, 2000), which enables easy genetic manipulation of the host methylation pathway

65   without the confounding influence of other DNA methyltransferases (*Dnmt1* and *Dnmt3*) present

66   in most other insect species (Group et al., 2010). While the role of *Dnmt2*-dependent methylation

67   is debated and multifaceted (Schaefer & Lyko, 2010; Raddatz et al., 2013; Takayama et al.,

68   2014), evidence demonstrates that the methylation machinery in *D. melanogaster* is not only

69   present but also functional (Lyko, Ramsahoye & Jaenisch, 2000; Kunert et al., 2003; Schaefer,

70   Steringer & Lyko, 2008; Gou et al., 2010). Moreover, overexpression of the mouse *Dnmt3a* in D.

71   melanogaster induces CI-like defects such as reduced rates of cell cycle progression and altered

72   chromosome condensation (Weissmann et al., 2003).

73   **Materials and Methods**

74   *Fly rearing and dissections*

75   All flies were reared on a cornmeal and molasses-based media at 25°C. The *Dnmt2* loss-

76   of-function mutant has been previously described (Goll et al., 2006). Briefly, the mutant contains

77   a 28bp insertion with multiple stop codons as well as a frameshift within the coding region of

78   *Dnmt2*. Overexpressing flies were created through the Gal4-UAS system. Crosses were

79   performed between virgin *nos*-Gal4 driver females (y^{1}w*; P{w[+mC]=GAL4-nos.NGT}40

80   (either *Wolbachia*-infected or uninfected) and 5-6 uninfected UAS-*Dnmt2* (Kunert et al., 2003),

81   UAS-GFP or *W^{1118}* males. Crosses for Supplemental Figure 1 were conducted between virgin

82   Act5c-Gal4 driver females (y^{1}w*;P{w[+mC]=Act5C-GAL4}25FO1/CyO,y$^+$, *Wolbachia* infected

83   or uninfected depending on desired progeny) and UAS-*Dnmt2* males. For Act5c-Gal4 crosses,

84   straight-winged progeny were assumed to be overexpressing *Dnmt2* while CyO expressing lines

85   were used as the wild-type expressing lines. *Wolbachia*-uninfected lines were created through

86   tetracycline treatment (20ug/mL for 3 generations) and infection status was confirmed through

87   PCR using the following primers: WolbF (GAAGATAATGACGGTACTCAC) and WolbR3

88   (GTCACTGATCCCACTTTAAATAAC) which target the 16S rRNA gene of *Wolbachia*. These

89   lines were further reared for at least three generations on undrugged media before

90   experimentation to avoid detrimental paternal effects seen in other systems (Zeh et al., 2012).

91   *w*Au, *w*No, and *w*Ri (also known as *w*Ri Agadir) strains of *Drosophila simulans* were

92   kindly provided by Charlat Sylvain (University Lyon, France).   All testes and ovary

93   dissections were performed in cold phosphate buffered saline (PBS). Males were dissected within

94   24 hours of emergence while females were aged 3-4 days before dissections. Testes samples

95   consisted of tissue obtained from a minimum of 20 males while ovary samples were pooled from

96   10 females each. Tissues were frozen and stored at -80°C before analysis.

97   *Hatch rate assays*

98   Assays were performed using a grape juice/agar media in 30mm plates for egg laying. For

99   each cross 32-48 individual crosses of one male and one female were set up in separate mating

100 chambers with individual grape juice plates. A minimal amount of a 1:2 dry yeast and water mix

101 was added to each plate and the parents were allowed to mate for 16 hours before the grape juice

102 plates were discarded. Fresh plates were then used for 24 hours, removed, and the number of eggs

103 laid was counted for each cross. The number of unhatched eggs was counted again at 36 hours

104 after the plates had been removed to determine hatch rates.

105 *MethylFlash quantification of DNA methylation*

106   Genomic levels of cytosine methylation (5-mC) were measured using the MethylFlash kit

107 (Epigentek; Farmingdale, NY, USA). 8-10 replicate sets of testes (20-40 testes pairs each

108 replicate) were dissected and DNA was isolated using the Puregene Tissue kit (Qiagen, Venlo,

109 Netherlands). 100ng of genomic DNA from each sample was used and each sample was analyzed

110 in duplicate on a BMG LabTech FLOUstar OPTIMA plate reader (Ortenberg, Germany)

111 according to manufacturer instructions.

112 *Wolbachia density*

113   Eight replicates each of whole animals (pools of 3), testes (pools of 20 pairs), and ovaries

114 (pools of 10 pairs) were collected and DNA was isolated. All males were less than 24 hours old

115 while females had been aged 3-4 days. Quantitative PCR was performed on a Bio-Rad CFX96

116 Real-Time System using iTaq Universal SYBR Green Supermix (Bio-Rad; Hercules, CA, USA).

117 *groEL* copy number, determined against a standard curve, was compared to counts for the host

118 gene *Actin*, also determined against a standard curve. It was assumed that one copy of *groEL* was

119 present in each *Wolbachia* genome and 1 or 2 copies of Act5c (for males and females,

120 respectively, as the gene is on the X chromosome) in each *Drosophila* genome. Primers: Act5c

121 (231bp product, Forward: ATGTGTGACGAAGAAGTTGCT Reverse:

122 GTCCCGTTGGTCACGATACC), groEL (97bp product, Forward:

123 CTAAAGTGCTTAATGCTTCACCTTC Reverse: CAACCTTTACTTCCTATTCTTG). qPCR

124 conditions: 50° 10 min, 95° 5 min, 40x (95° 10 sec, 55° 30 sec), 95° 30 sec. Followed by melt

125 curve analysis (0.5° steps from $65\text{-}95^\circ$ for 5 sec each).

126 *Gene expression*

127   Quantitative PCR was performed on a Bio-Rad CFX96 Real-Time System using iTaq

128 Universal SYBR Green Supermix (Bio-Rad; Hercules, CA, USA). RNA was isolated from 8 sets

129 of testes (20 pairs each) using the RNeasy Mini kit (Qiagen; Venlo, Netherlands) and DNA was

130 removed with the TURBO DNA-free DNase kit (Ambion; Grand Island, NY, USA). cDNA was

131 synthesized using a SuperScript III First-Strand kit (Invitrogen; Grand Island, NY, USA) and

132 diluted 1:20. All calculations were done using delta delta Ct with Rp49 expression used for

133 normalization of results. Primers: *Dnmt2* (150bp product, Forward:

134 CCGTGGCGTGAAATAGCG Reverse: ACACCGCTTTCGGAGGACG), Rp49 (154bp product,

135 Forward: CGGTTACGGATCGAACAAGC Reverse: CTTGCGCTTCTTGGAGGAGA). qRT-

136 PCR conditions are the same as used in qPCR for *Wolbachia* densities.

137 *Bisulfite sequencing*

138   100 testes were dissected in PBS from *Wolbachia* infected (y^1w^*) and uninfected males

139   and flash frozen. gDNA was then isolated using the Puregene kit (Qiagen; Venlo, Netherlands)

140   and fragmented by Covaris shearing. gDNA was submitted to Vanderbilt Technologies for

141   Advanced genomics (VANTAGE) where the PE-75 bp library was generated using the TruSeq

142   sample preparation kit (with methylated adapters), bisulfite treated, PCR amplified (EpiMark and

143   ZymoTaq) and sequenced (Illumina HiSeq 2000, 86bp PE read). Sequences with $\geq$10x coverage

144   were analyzed using Bismark (Krueger & Andrews, 2011) and cytosines which were methylated

145   in at least one read were counted.

146   **Results and Discussion**

147   ***Wolbachia* *w*Mel increases levels of testes DNA methylation**

148   MethylFlash analysis of host DNA from testes revealed that infection with the *Wolbachia*

149   strain *w*Mel in *Drosophila melanogaster* increases levels of genome-wide cytosine methylation

150   (Figure 1a). More importantly, this methylation is specific to the host testes (55% increase, P =

151   0.0015, Mann Whitney U test) and is not observed in the ovaries, consistent with the prediction

152   that only the paternal genome is modified during cytoplasmic incompatibility. The overall levels

153   of methylation are extremely low, which is consistent with previously reported levels of

154   methylation in *Drosophila melanogaster* (Lyko, Ramsahoye & Jaenisch, 2000; Kunert et al.,

155   2003). Conflicting reports over the strength and prevalence of DNA methylation in *D.*

156   *melanogaster* (Lyko et al., 2000; Raddatz et al., 2013; Schaefer and Lyko, 2010) led us to test the

157   validity of our initial results with genome-wide bisulfite sequencing. Results indicate that,

158   contrary to most other species, DNA methylation in *Drosophila melanogaster* is not CpG specific

159   and is evenly distributed over cytosine residues (Figure 1b, Table S1). Sequencing results also

160   mirror those of MethylFlash and show that infection with *w*Mel increases testes DNA

161   methylation 46% across all cytosine residues with a range of 43-54% depending upon the type of

162   cytosine residue (CpG, CHG, or CHH) (Figure 1b, Table S1). The minor discrepancies between

163   MethylFlash and bisulfite sequencing (55% and 46% increase in methylation, respectively) are

164   likely due to the sensitivity of the MethylFlash system on such low quantities of methylation. A

165   more thorough investigation of the bisulfite sequencing, including changes in promoter and gene

166   body methylation, is ongoing.

167   **Overexpression of *Dnmt2* neither induces nor strengthens CI**

168   *Drosophila melanogaster* possess just one canonical DNA methyltransferase, *Dnmt2*, and

169   overexpression of this enzyme in fruit flies has previously been shown to increase levels of DNA

170   methylation (Kunert et al., 2003; Schaefer, Steringer & Lyko, 2008). Utilizing the Gal4-UAS

171   expression system, we overexpressed *Dnmt2* in uninfected males to test if an increase in host

172   methylation alone could induce the CI defect of reduced embryo hatching rates. Figure 2 shows

173   that there was no discernable difference in hatching rates with uninfected males expressing

174   increased or wild type levels of *Dnmt2* (P = 0.91, MWU). The result was confirmed using an

175   Actin-based driver that again yielded no discernable differences in hatch rates compared to wild

176   type flies (Figure S1, P = 0.83, MWU). These findings specify that amplified levels of *Dnmt2*-

177   mediated epigenetic regulation are not sufficient to recapitulate cytoplasmic incompatibility.

178   If multiple factors are responsible for CI, it is possible that overexpression of *Dnmt2*,

179   while unable to induce CI in uninfected flies, may be able to strengthen the modification in the

180   presence of *Wolbachia*. To test this hypothesis, we overexpressed *Dnmt2* in *Wolbachia*-infected

181   males that were then mated to uninfected virgin females. Surprisingly, *Dnmt*2 overexpression in

182   males decreased the level of cytoplasmic incompatibility by an average of 17.4% (Figure 3). This

183   effect is not dependent on the *Dnmt*2 expression status of the female and suggests that increased

184   methylation of host DNA can diminish the penetrance of cytoplasmic incompatibility.

185   **Overexpression of *Dnmt*2 reduces *Wolbachia* titers in host testes**

186   Previous work suggested that *Dnmt*2 is detrimental to *Wolbachia* proliferation in

187   mosquitoes. In fact, *Wolbachia* strain *w*MelPop-CLA utilizes a host miRNA to downregulate

188   *Dnmt2* expression when infecting *Aedes aegypti* (Zhang et al., 2013). We observed no differences

189   in Dnmt2 expression between *w*Mel infected and uninfected *D. melanogaster* testes (data not

190   shown) but hypothesized that overexpression of *Dnmt2* in the host may adversely affect

191   *Wolbachia* titers. In support of this prediction, we found that *Wolbachia* density (as measured by

192   the ratio of *Wolbachia groEL* gene copy number / *Drosophila Actin* gene copy number)

193   decreased by 17.3% in adult testes overexpressing *Dnmt2* transcripts by 9.6% (Figure 4 and

194   Figure S2, respectively). The low level of transcript overexpression could be specific to the

195   developmental stage of the experimental sample or due to usage of a pUAST vector for germline

196   expression instead of the more efficient pUASP (Kunert et al., 2003). While the upregulation of

197   *Dnmt2* in infected males is not statistically significant, it remains possible that actual protein

198   levels are much higher than those represented by RNA transcripts. Strong protein expression, as

199   measured by Western blot, was seen in uninfected ovaries (data not shown) Nevertheless, the

200   17.3% decrease in *Wolbachia* titers compares well with the 17.4% reduction in CI penetrance

201   reported above. Expression of the negative control green fluorescent protein (GFP) did not reduce

202   *Wolbachia* titers, as expected (Figure S3). As the bacterial and phage density models of CI

203   specify that *Wolbachia* titers in the testes are linked to the strength of CI (Breeuwer & Werren,

204   1993; Bordenstein et al., 2006), we conclude that the reduction of CI observed in *Dnmt2-*

205   overexpressing males is likely due to reduced *Wolbachia* density.

206   Even though we do not observe any change in *Dnmt2* mRNA levels after *Wolbachia*

207   infection, we cannot rule out that *Wolbachia* may be affecting intracellular *Dnmt2* localization

208   rather than levels of gene expression. An increase in localization of *Dnmt2* to the nucleus would

209   not only protect the cytosolic *Wolbachia* but also explain the additional genomic methylation

210   associated with infection. In this scenario, the testes-specific increase in host methylation initially

211   observed would simply be a by-product of high *Wolbachia* activity. Additionally, an

212   immunomodulatory role for *Dnmt2* in *Drosophila* has already been documented in protection

213   against RNA viruses (Durdevic et al., 2013) though we believe the findings in this report are the

214   first evidence for a putative antibacterial role for *Dnmt2* in fruit flies.

215   **Hosts defective in DNA methylation still exhibit CI**

216   As *Dnmt2* overexpression did not induce nor increase cytoplasmic incompatibility, we

217   next tested the strength of CI in hosts defective in the methyltransferase pathway. Knockout

218   mutants for *Dnmt2* characterized by Goll et al (Goll et al., 2006) were acquired and found by

219   PCR and amplicon sequencing to be infected by the *w*Mel strain of *Wolbachia*. The strain is

220   hereafter referred to as Mut and was tetracycline treated for 3 generations to create the uninfected

221   line MutT. We show by MethylFlash that the increase in host DNA methylation induced by

222   *Wolbachia* infection is abolished in the knockout Mut background (Figure S4) and is thus *Dnmt2-*

223   dependent. However, loss of this crucial enzyme in the DNA methylation pathway has no effect

224   on the penetrance of CI (Figure 5), as shown in comparisons between mutant and wild type males

225   mated to uninfected females (P = 0.13, MWU). The low level of DNA methylation still present in

226   mutants has recently been observed by others (Boffelli, Takayama & Martin, 2014) and suggests

227   a possible mechanism of DNA methylation in *Drosophila* that is independent of canonical DNA

228   methyltransferases. Thus, it is possible that CI could be induced by alterations in genomic

229   methylation but in a *Dnmt2*-independent manner.

230   Curiously, despite the previously observed role for *Dnmt2* in host immunity (Zhang et al.,

231   2013; Durdevic et al., 2013), the mutants observed here exhibit no increase in *Wolbachia* titers

232   within any of the tissues tested (Figure S5). It is interesting to note that *Dnmt2* mutant

233   *Drosophila*, derived from the W^{1118} background line, have titers that are, on average, half of those

234   seen in y^{1}w* background lines (see Figure 4). This difference has been observed several times in

235   our experiments and suggests either a differing ability of the host lines to control *Wolbachia* titers

236   or an as yet unclassified difference in the *w*Mel strains infecting these flies.

237   **Host levels of DNA methylation do not correlate with strength of CI**

238   To substantiate the claim that DNA methylation is not involved in the induction of CI for

239   other *Wolbachia* strains and/or host species, we tested the DNA methylation status of testes DNA

240   from *Drosophila* species infected with various strains of *Wolbachia*. These taxa include *D.*

241   *simulans* infected with strains *w*Ri, *w*No, and *w*Au, which express strong, moderate, and no CI,

242   respectively. We also tested a different *D. melanogaster*-infecting strain *w*Mel derived from the

243   W^{1118} background strain instead of y^{1}w*. As previously mentioned, while the W^{1118} line induces

244   strong CI, *Wolbachia* titers are much lower in these animals compared to the infection found in

245   y^{1}w*.

246   Results show that methylation status of the infected host testes is random with regards to

247   the strength of CI (Figure 6). While infection with the high CI-inducer *w*Ri exhibits higher

248   methylation in infected testes as compared to uninfecteds, this effect is marginally insignificant

249   (P = 0.072, MWU) and is countered by data from the *w*Au strain, which causes no CI but still

250   significantly increases host DNA methylation in testes (P = 0.0047, MWU). Furthermore,

251   infection with the *w*No strain of *Wolbachia*, which causes moderate CI, actually has less

252   methylation in host testes. Finally, a low-titer infection of *w*Mel (W^{1118}), while still inducing CI,

253   does not induce the same level of DNA methylation associated with a high-density infection

254   (y^{1}w*).

255   **Conclusions**

256   The underlying mechanism of *Wolbachia*-induced CI largely remains elusive after several

257   decades of research. Here we show that host DNA methylation, a promising candidate pathway

258   hypothesized to play a role (Negri, 2011; Saridaki et al., 2011; Ye et al., 2013b; Liu et al., 2014),

259   does not seem to be involved in the induction of cytoplasmic incompatibility. While *Wolbachia*

260   infection preferentially increases host DNA methylation in *Drosophila melanogaster* testes

261   (Figure 1), this modification is not conserved across other CI-causing strains of *Wolbachia*

262   (Figure 6) and overexpression of a host methyltransferase neither induces nor increases rates of

263   CI. We have also found that *Wolbachia*-induced changes in host methylation are dependent on

264   the DNA methyltransferase *Dnmt2* (Supplemental Figure S4) but that *Drosophila melanogaster*

265   lacking *Dnmt2* still suffer from CI (Figure 5). Finally, we found *Dnmt2* has anti-*Wolbachia*

266   properties, as previously reported in *Aedes aegypti* (Zhang et al., 2013), and overexpression of

267   *Dnmt2* reduces the strength of CI.

268   Taken together, we show that one of the canonical chromatin modification pathways,

269   *Dnmt2*-dependent DNA methylation, likely has no role in *Wolbachia*-induced cytoplasmic

270   incompatibility. *Wolbachia* infection can be associated with changes in host methylation levels,

271   but it is most likely a consequence of the bacteria modulating host immune response or the host

272   defending itself against the infection. The possibility also remains that infection alters gene-

273   specific, and *Dnmt2*-independent, levels of methylation that our current study of genomic

274   methylation levels has not detected. While further investigation of the *Dnmt2* epigenetic pathway

275   will not elucidate a CI mechanism, it may be useful in studying the complex nature of pathogen-

276   host interactions between *Wolbachia* and the many species it infects. It remains possible that a

277   novel methyltransferase, recently suggested to exist in *Drosophila* (Takayama et al., 2014;

278   Boffelli, Takayama & Martin, 2014), could affect CI.

279   **Acknowledgements:**

280   We are grateful to Rini Pauly for computational assistance and four reviewers and the

281   editor for constructive feedback.

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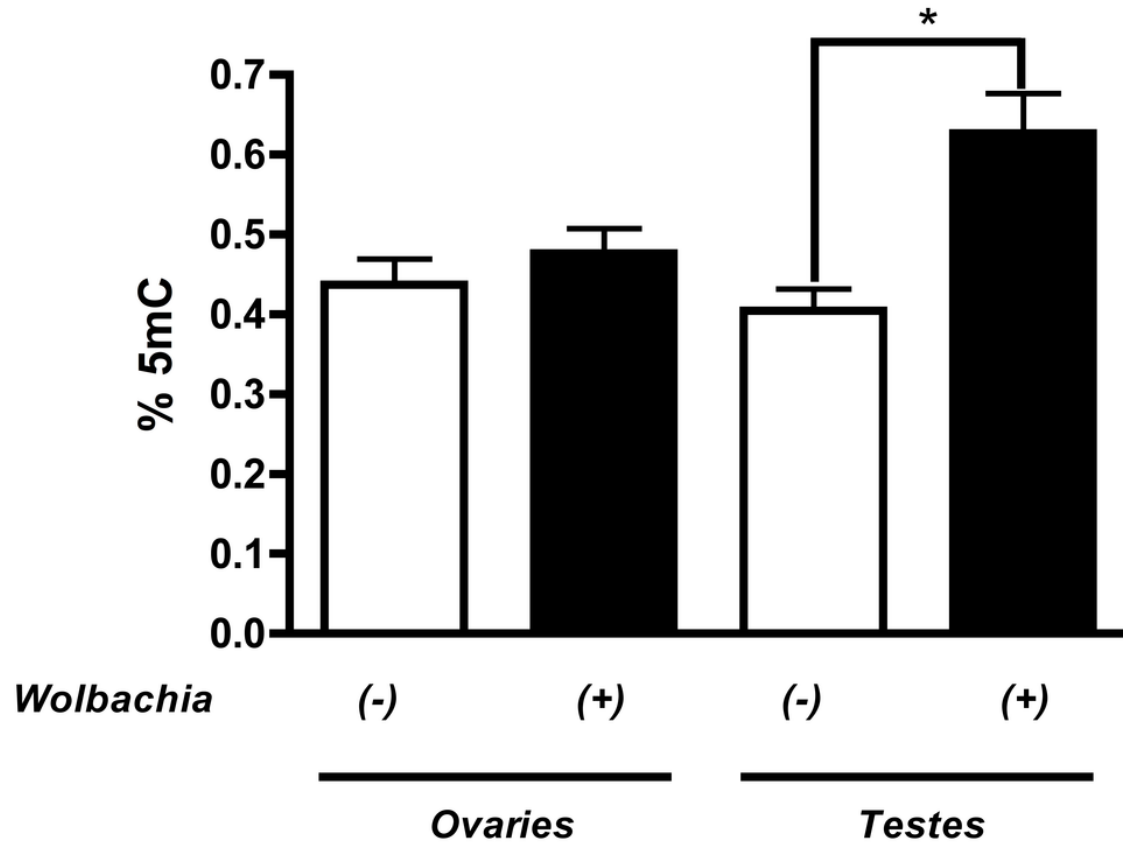
Figure 1

Wolbachia increase host levels of DNA methylation.

(a) *Wolbachia* infection (*w*Mel) of *Drosophila melanogaster* increases DNA methylation in host testes by 55% (P = 0.0015, Mann-Whitney U (MWU) test, two-tailed), as measured by the ELISA-based MethylFlash kit. This increase is not observed in host ovaries (P = 0.25). Bars denote standard error of the mean (SEM) (b) Bisulfite sequencing of *Drosophila melanogaster* testes DNA shows that infection by *w*Mel increases methylation of all cytosine residues including CpG (43%), CHG (54%), and CHH (50%).

a)

b)

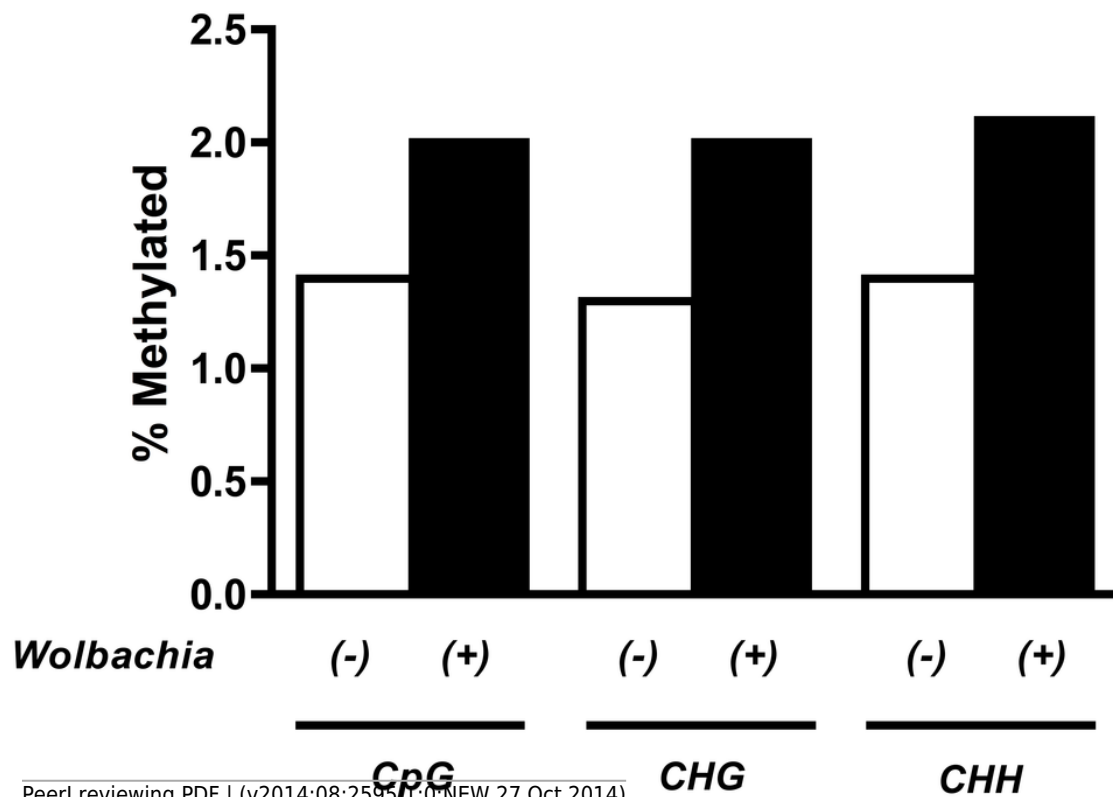



Figure 2

Expression of DNA methyltransferase 2 does not induce CI.

Overexpression of the DNA methyltransferase *Dnmt2* in uninfected males, utilizing the Gal4-UAS system with a *nos* driver, does not reduce hatching rates. Dnmt2 = overexpressing flies; WT = wild type flies. Bars denote standard error of the mean (SEM).

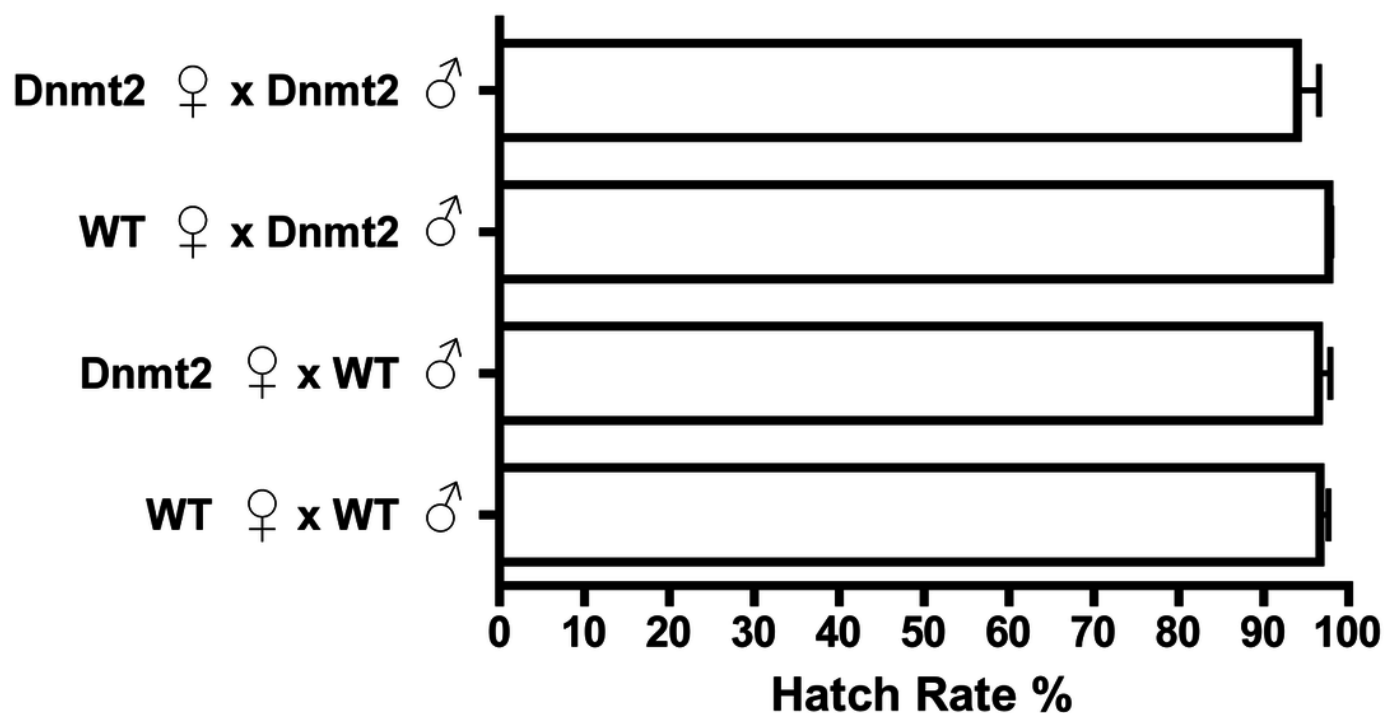



Figure 3

Overexpression of *Dnmt2* reduces levels of CI.

The overexpression of *Dnmt2* in *Wolbachia*-infected males decreases rates of CI (P < 0.05, Mann-Whitney U test). *Dnmt2* expression in the mother has no affect. Bars denote standard error of the mean (SEM). Dnmt2 = overexpressing flies; WT = wild type flies.

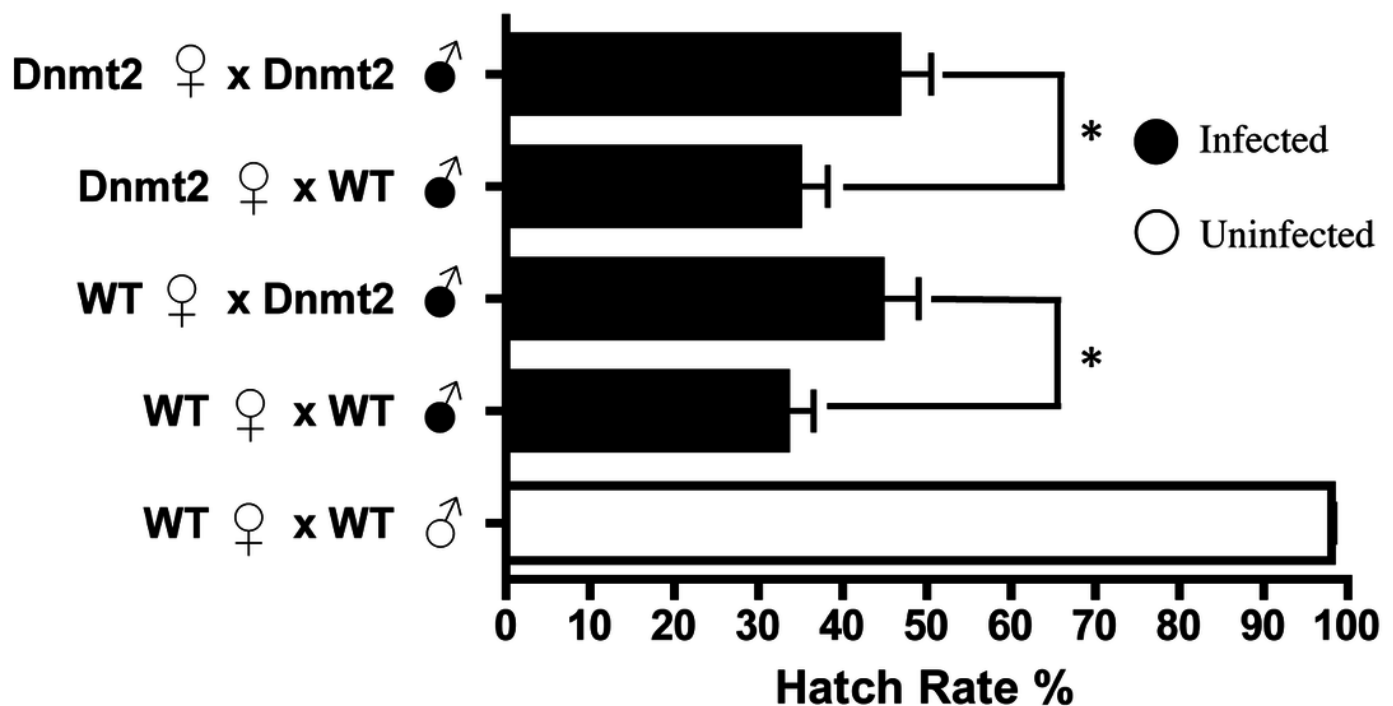



Figure 4

Dnmt2 overexpression alters *Wolbachia* titers.

Overexpression of *Dnmt2* reduces *Wolbachia* titers within the testes (P < 0.01, MWU test) but has no affect on titers within ovaries or whole flies. *Wolbachia* infection is derived from the y[1]w[*] *Drosophila* background. Bars denote standard error of the mean (SEM). Dnmt2 ++ = overexpressing flies; Dnmt2 + = wild type flies. (P = 0.007, Mann-Whitney U test, two-tailed).

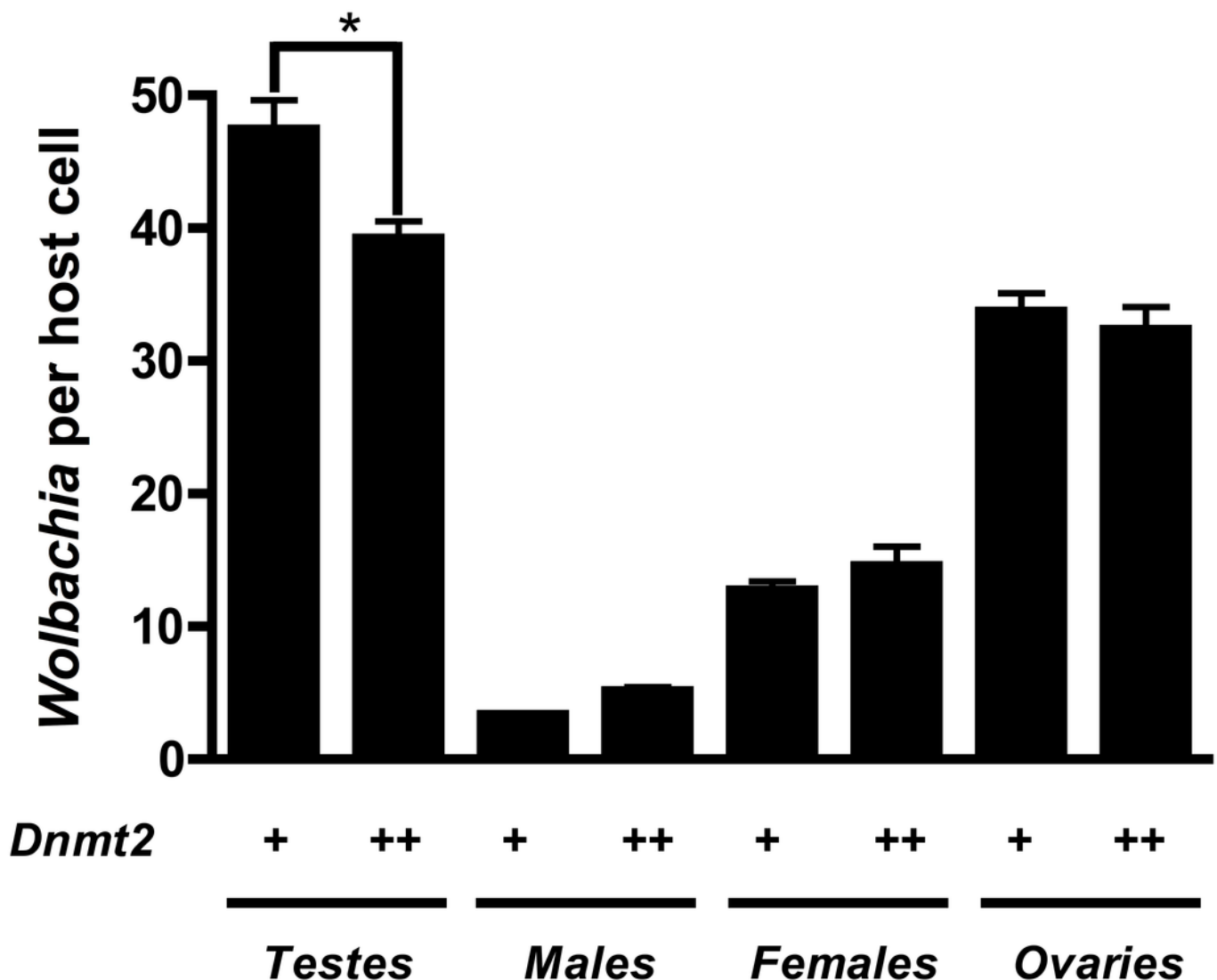



Figure 5

Dnmt2 mutants express wild-type levels of CI.

Crosses with *Dnmt2*-mutant males ("Mut") show that *Dnmt2* expression within the father is not necessary for expression of CI. Mut = Dnmt2 mutant flies; WT = wild type flies. Bars denote standard error of the mean (SEM).

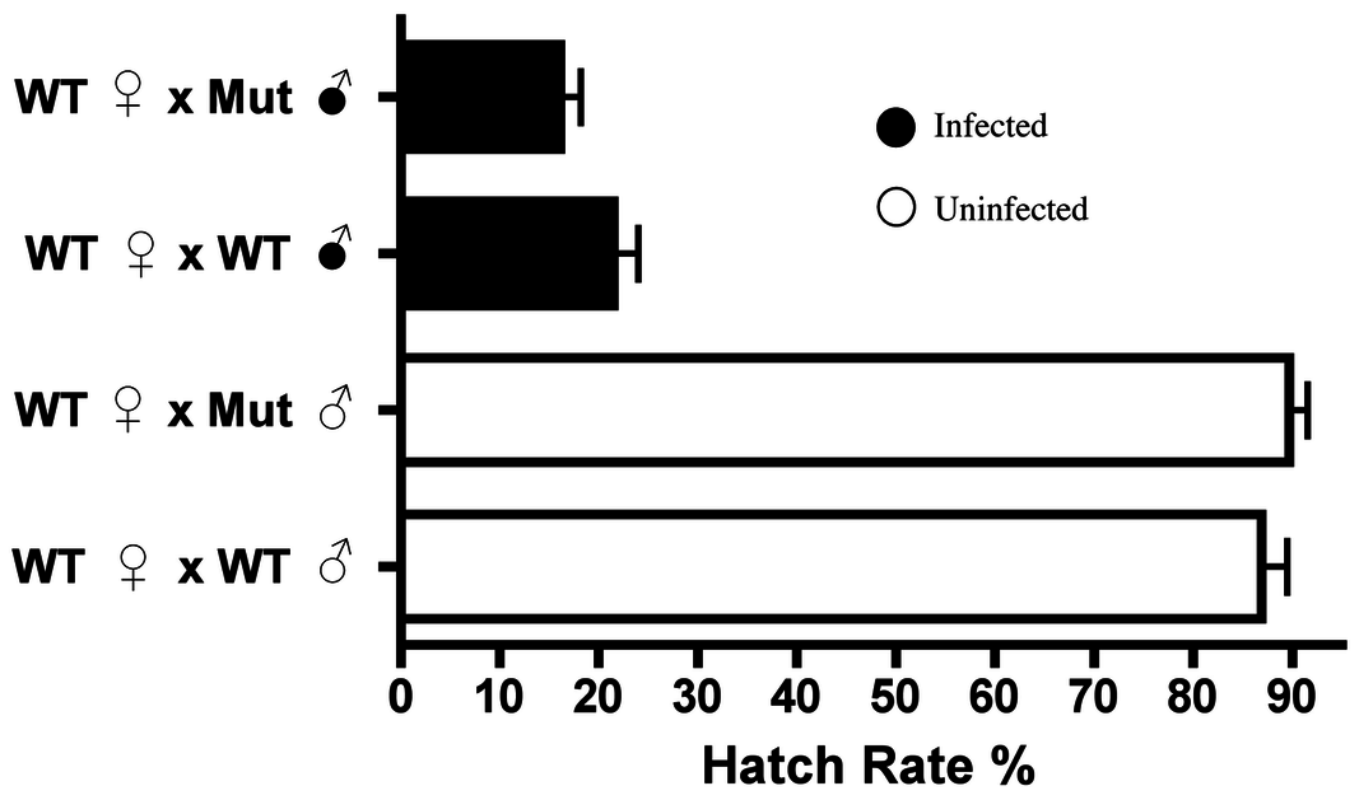



Figure 6

Levels of host DNA methylation do not correlate with strength of CI.

Testing of several different *Wolbachia* infections, capable of inducing various levels of CI in their respective hosts, shows that levels of host DNA methylation and strength of CI are not correlated. Bars denote standard error of the mean (SEM) of testes DNA methylation, as measured by MethylFlash. White bars (-) denote uninfected flies and black bars (+) denote infected flies. # indicates levels of methylation too low for detection.