Reply

Validating the Demethylating Effects of 5-aza-2′-deoxycytidine in Insects Requires a Whole-Genome Approach

(A Reply to Ellers et al.)

Nicola Cook,[1,*,†] Darren J. Parker,[2,*] Eran Tauber,[3] Bart A. Pannebakker,[4] and David M. Shuker[1]

1. School of Biology, University of Saint Andrews, Saint Andrews, United Kingdom; 2. Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland; and Swiss Institute of Bioinformatics, Lausanne, Switzerland; 3. Faculty of Natural Sciences, University of Haifa, Haifa, Israel; 4. Department of Plant Sciences, Laboratory of Genetics, Wageningen University, Wageningen, The Netherlands

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abstract: We previously demonstrated that treatment with the demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) alters the offspring sex ratios produced by females of the parasitoid wasp *Nasonia vitripennis*. Females allocate offspring sex ratio in line with local mate competition theory, producing more or less female-biased sex ratios as the number of other females laying eggs on a patch varies, thereby reducing competition among their sons for mates. Interestingly, treatment with 5-aza-dC did not ablate the facultative sex allocation response. Instead, sex ratios became less female biased, a shift in the direction of the optimum sex ratio for paternally inherited alleles according to genomic conflict theory. This was the first (albeit indirect) experimental evidence for genomic conflict over sex allocation. In their comment, Ellers and colleagues assayed the effects of 5-aza-dC on DNA methylation in 10 *Nasonia* genes, finding no evidence of demethylation in these 10 genes, from which they conclude that 5-aza-dC has no demethylating capability in *N. vitripennis*. Quantifying the efficacy of 5-aza-dC in terms of demethylation is indeed crucial to in-depth interpretation of studies using 5-aza-dC to link phenotypes to epigenetic regulation. Here we outline the mode of action of 5-aza-dC and demonstrate that determining the efficacy of 5-aza-dC in insect systems requires a whole-genome approach.

Keywords: sex ratio, 5-aza-2′-deoxycytidine, DNA methylation, *Nasonia vitripennis*.

DNA Methylation, Sex Allocation, and 5-aza-2′-deoxycytidine

In a previous study, we demonstrated that treatment with the demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) alters the sex allocation response of *Nasonia vitripennis* females (Cook et al. 2015). As with all Hymenoptera, *N. vitripennis* is a haplodiploid species, with males arising from unfertilized, haploid eggs and females developing from fertilized, diploid eggs. Females are in putative control of sex allocation, either laying unfertilized eggs that will become sons or releasing sperm to fertilize eggs that will become daughters. Female *Nasonia* allocate sex broadly in line with local mate competition (LMC) theory (Hamilton 1967; Burton-Chellew et al. 2008; West 2009); females laying eggs alone will produce very female-biased sex ratios to minimize competition among their sons for mates. As the number of laying females (foundresses) on a patch increases, though, the level of LMC consequently declines and less female-biased sex ratios are produced.

Recent theory, however, has demonstrated that maternally and paternally inherited alleles should have differing patterns of optimal sex allocation under LMC, with maternally inherited alleles selected to produce more female-biased sex ratios (for a detailed explanation, see Wild and West 2009). Importantly, parent-of-origin information must be associated with each allele, for instance, via epigenetic marks such as DNA methylation. We demonstrated that disrupting DNA methylation with 5-aza-dC did not ablate the facultative sex allocation response but instead found that sex ratios were shifted in the direction of the paternal optimum (sex ratios became less female biased; Cook et al. 2015). This result was consistent with predictions from genomic conflict over sex allocation theory (Wild and West 2009), suggesting that unmanipulated sex ratios are closer to the optimum for maternally inherited alleles (fig. 1). Ellers et al. (2019), in their valuable comment on this study, raised the valid point that the efficacy of 5-aza-dC in insect systems should be tested and have suggested from their own experimentation that 5-aza-dC does not disrupt methylation in *N. vitripennis*. To fully address their comments, we will begin by briefly reviewing

* These authors contributed equally to this work.
† Corresponding author; email: nc52@st-andrews.ac.uk.
ORCIDs: Cook, https://orcid.org/0000-0003-4462-0116; Parker, https://orcid.org/0000-0003-4027-7779; Tauber, https://orcid.org/0000-0003-4018-6535; Pannebakker, https://orcid.org/0000-0001-8503-3896; Shuker, https://orcid.org/0000-0002-2092-6236.

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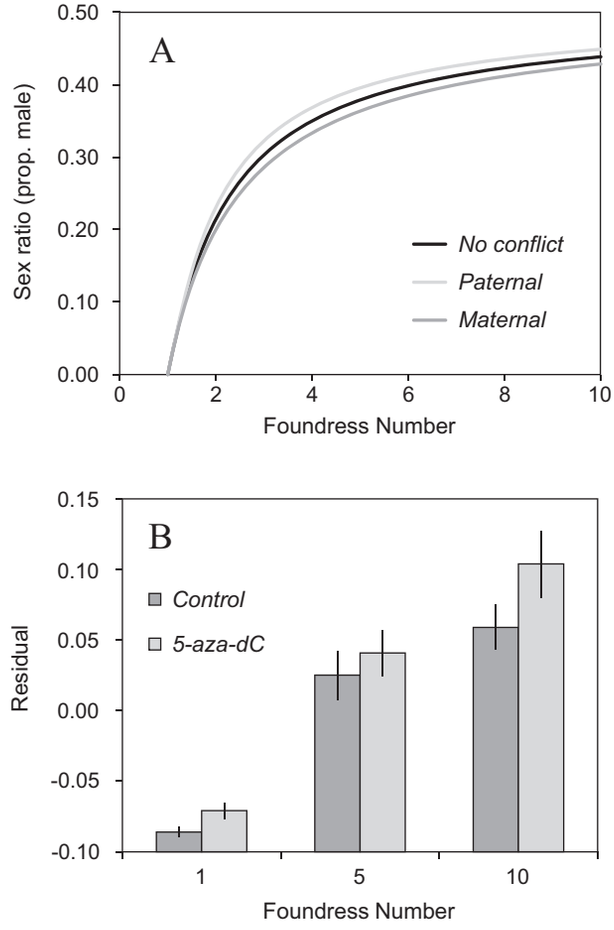

Figure 1: Reproduced from Cook et al. (2015). *A*, Optimal sex ratios under local mate competition (LMC) for maternally inherited alleles (dark gray) and paternally inherited alleles (light gray) for a haplodiploid species with female control of sex allocation. Shown in black is the LMC prediction with no genomic conflict. Equations are shown in Wild and West (2009). *B*, Females treated with 5-aza-2′-deoxycytidine (5-aza-dC; light gray bars) produce slightly less female-biased sex ratios than controls (dark gray bars). Both treated and untreated females vary sex ratio with foundress number as predicted. Data are presented as residuals after controlling for experimental replicate. Error bars are binomial confidence intervals.

the mechanism by which 5-aza-dC brings about the demethylation of DNA, before showing that assessing the role of 5-aza-dC requires a whole-genome approach.

Originally synthesized in 1964 by Piskala and Sorm (1964; and also known as 5-AZA-CdR, DAC, decitabine, ZdCyD, and AzaD, among other names), 5-aza-dC is an analog of the natural nucleoside 2′-deoxycytidine (the carbon at the 5-position of the pyrimidine ring is replaced by a nitrogen) and was repurposed as a demethylating agent in 1980 (Jones and Taylor 1980). Much of the current attention surrounding 5-aza-dC is attributable to its success in the treatment of cancers, particularly acute myeloid leukemia and myelodysplastic syndrome (Bryan et al. 2011; Momparler 2012; Seelan 2018). In mammals, DNA methylation acts as a transcriptional silencer, and the clinical efficacy of 5-aza-dC results from its ability to reactivate tumor-suppressor genes silenced by aberrant DNA methylation (Karahoca and Momparler 2013).

DNA methylation for the vast majority of cases occurs at the C5 position of the cytosine ring in CpG dinucleotides and is catalyzed by DNA methyltransferase enzymes (Dnmts), of which three types are known in eukaryotes. Dnmt3 establishes DNA methylation patterns de novo, Dnmt1 maintains these patterns, and Dnmt2 is involved in tRNA methylation (Lyko 2018). DNA methyltransferase genes have been found across the Insecta, varying in presence/absence and copy number across species. The *N. vitripennis* genome encodes a full DNA methylation toolkit, including three copies of Dnmt1 (Werren et al. 2010). DNA methylation itself is also present across the Insecta and is largely restricted to the transcribed regions of genes, in contrast to DNA methylation in mammals, which is found throughout the genome (Glastad et al. 2011; Bewick et al. 2017). In addition, genomic levels of DNA methylation in insects are much lower (0%–14%; Bewick et al. 2017) as compared to 60%–90% in mammals (Glastad et al. 2011). Regardless of the level of DNA methylation present within a genome or whether methylation signals transcriptional suppression (as in mammals) or acts to maintain transcript integrity and/or initiate/regulate splicing (as has been suggested for the insects; reviewed in Glastad et al. 2011), the epigenetic modification itself is, to our knowledge, chemically identical.

The introduction of 5-aza-dC to a cell results in a cascade of chemical reactions to cause an eventual reduction in DNA methylation. On its introduction to a cell, 5-aza-dC is activated in situ via phosphorylation by deoxycytidine kinase, producing the biologically active triphosphate form of 5-aza-dC, 5-aza-dCTP (Seelan et al. 2018). This active form is an excellent substrate for DNA polymerase and is readily incorporated into DNA during the S-phase of the cell cycle (Bouchard and Momparler 1983; Christman et al. 1983; Christman 2002; Momparler 2005). In the resulting daughter strands produced from a DNA replication event, hemimethylated CpG sites are remethylated by Dnmt1 to fully restore the patterns of methylation present in the parent strand. However, where 5-aza-dCTP is incorporated into the daughter DNA strand, a covalent bond is formed between 5-aza-dCTP and a cysteine residue in the active site of Dnmt1. Since the carbon at the 5-position in dCTP is replaced with a nitrogen in 5-aza-dCTP, the methyl transfer reaction cannot take place and Dnmt1 becomes irreversibly bound to the DNA (Creusot et al. 1982; Taylor and Jones 1982; Seelan et al. 2018). Thus, treatment with 5-aza-dC results in a diminishing pool of Dnmt1, resulting in the passive loss of DNA methylation; this is the key effect of 5-aza-dC. Importantly, there

is evidence that the effects of 5-aza-dC are unlikely to be random across methylated CpGs in a given genome, as genomic context can influence the extent to which its action is effective. DNA sequence context plays a role, with demethylation of CpGs outside of CpG islands occurring at a higher frequency (Mossman et al. 2010; Hagemann et al. 2011). The distribution of transcription factor binding sites is also influential with, for example, "demethylation-sensitive" genes enriched for forkhead box (FOX) binding sites and "demethylation-resistant" genes enriched for basic helix-loop-helix (bHLH) binding sites. Indeed, some genes are never demethylated, and these are enriched for binding motifs not present in demethylated genes. More broadly, 5-aza-dC activity appears to be targeted to transcriptionally active euchromatin in mammalian cells (Ramos et al. 2015). Given that the mode of action of 5-aza-dC is passive, these results indicate that specific loci remain demethylated after replication, whereas others at first appear resistant because they become preferentially remethylated by the remaining pool of DNA methyltransferase, leading to nonrandom patterns of demethylation (Hagemann et al. 2011).

We agree with Ellers et al. (2019) that quantifying the demethylation efficacy of orally administered 5-aza-dC in *N. vitripennis* will permit a more complete understanding of the relationship between DNA methylation and the sex allocation phenotype in *Nasonia* (see Cook et al. 2015). More broadly, this will allow us to determine the extent to which genome-wide demethylation can be altered with 5-aza-dC in insect systems. In their analysis of the involvement of DNA methylation in the photoperiodic diapause response in *Nasonia*, Pegoraro et al. (2016) demonstrated that consumption of 5-aza-dC led to demethylation at specified CpG sites on a small scale. Ellers et al. (2019) have examined the demethylating effect of 5-aza-dC at 155 CpG sites spread across nine candidate genes in a slightly more comprehensive analysis. The authors found no significantly differentially methylated CpGs at any of the 155 sites in association with 5-aza-dC exposure and conclude from this result that 5-aza-dC has no demethylating effect. To our knowledge, there is no evidence to suggest that the candidate genes chosen by Ellers et al. (2019) are more (or less) likely to be influenced by 5-aza-dC—that is, to have genomic contexts that favor or disfavor its action. It would also be the first time that 5-aza-dC would have been shown to be inert in an organism in terms of influencing DNA methylation. As such, to get a more complete picture and to more accurately quantify the demethylation efficacy of 5-aza-dC, we suggest that a whole-genome approach is required.

In the first instance, it is important to note that genomic DNA methylation in *Nasonia* is relatively low, with estimates ranging from 0.63% to 1.6% of all CpG sites methylated (Wang et al. 2013; Beeler et al. 2014). These estimates are comparable with other hymenopterans, such as the honey bee, which has approximately 0.7% methylated CpGs (Lyko et al. 2010). Therefore, a demethylation effect will be inherently difficult to detect just by looking across a small number of CpGs. Ellers et al. (2019) examined fragments of nine genes to examine for evidence of demethylation due to 5-aza-dC treatment. Two genes were chosen because of a heavy methylation pattern (*aa* and *rnapol*), and five (*acc*, *fabd*, *fasn1*, *fasn3*, and *mcd*) are part of the fatty acid metabolic pathway, purported to be epigenetically regulated. Two additional genes were selected from the study of Pegoraro et al. (2016; *perq* and *wdr36*) based on the incorrect assumption that these genes are heavily methylated under the conditions tested by Ellers and colleagues. In fact, *perq* and *wdr36* display elevated methylation only under long photoperiods. While these genes may be heavily methylated under certain conditions and/or epigenetically regulated, there is no confirmed evidence in insects that the genomic context of these particular genes predisposes them to demethylation by 5-aza-dC. As outlined above, demethylation via 5-aza-dC occurs due to a lack of functional Dnmt1 to restore methylation to CpG sites postreplication. Given the contextual nature of the action of 5-aza-dC, there is a risk of false negatives in terms of assessing its genome-wide action. Therefore, a lack of demethylation in the gene fragments examined by Ellers et al. (2019) cannot be used to conclude that 5-aza-dC does not affect DNA methylation across the genome more broadly.

Quantifying the Efficacy of 5-aza-dC

Recently, we carried out a genome-wide analysis of the demethylating capacity of 5-aza-dC in *Nasonia vitripennis* (Cook et al. 2018). Briefly, female wasps were exposed to one of three 5-aza-dC exposure regimes: (1) 20% sucrose for 24 h, (2) 20% sucrose supplemented with 10 μM of 5-aza-dC for 24 h, and (3) 20% sucrose supplemented with 10 μM of 5-aza-dC for 48 h. For each of these three regimes, females were harvested at 0, 24, and 48 h postexposure, and prior to DNA extraction, heads were excised from the bodies as a nod in the direction of a tissue-specific analysis. Whole-genome sequencing of bisulphite-treated DNA samples was carried out to determine the methylation status of individual CpG sites across the genome (proportion of methylated reads/total reads per CpG). Median coverage depth across the whole experiment was approximately 900 times across 4 million CpGs after quality filtering.

To examine the data as a whole, we summed the proportion of methylated reads for each CpG by gene to broadly account for contextual similarity of CpGs within the same genic region. Data were available for over 11,000 genes comprising 85% of the protein-coding genes in the *N. vitripennis* genome. Distinct clustering of samples was associated with the 5-aza-dC exposure regime, clearly demonstrating that 5-aza-dC exposure has a quantifiable effect on DNA methylation

status on a genome-wide scale (fig. 2*A*). To explore DNA methylation changes at the gene level, we used a generalized linear modeling approach to determine which genes had significantly different proportions of methylation across the gene with respect to 5-aza-dC exposure regime, time point after exposure, and tissue. To encapsulate these results, we found that approximately 8,500 genes showed significantly altered methylation in response to treatment with 5-aza-dC. Interestingly, it appears that methylation in some genes initially increases in response to treatment with 5-aza-dC, an effect that is noticeable after 24 h of exposure and then less prevalent after 48 h of exposure (fig. 2*B*). In our data, two of the genes tested by Ellers and colleagues, *aa* and *rnapol*, do not exhibit significant differential methylation in association with feeding regime in our experiments. However, four of their tested genes (*perq*, *acc*, *fasn1*, and *fasn3*) exhibit significantly altered methylation levels in association with an overall effect of feeding regime. Interestingly, a decrease in methylation levels of these genes (relative to controls) is noted in wasps that were exposed to 5-aza-dC for a 48-h period but not in wasps exposed to 5-aza-dC for a 24-h period. This indicates that 5-aza-dC may take longer to exert its effects on these four genes. We also note that Ellers and colleagues have used newly emerged *N. vitripennis* females, whereas we used 2-day-old mated females; the effects of 5-aza-dC are known to differ with life stage (see below). We are unable to draw comparisons between our analysis and that of Ellers and colleagues for their remaining three genes; for *wdr36* and *fabd* we do not find any CpGs in the Ensembl *N. vitripennis* annotation (ver. 2.1), and *mcd* was excluded from our analysis as genes with zero coverage for all samples in any particular treatment group were removed prior to statistical analysis.

Hypermethylation has been observed previously in response to treatment with 5-aza-dC. Using amplification of

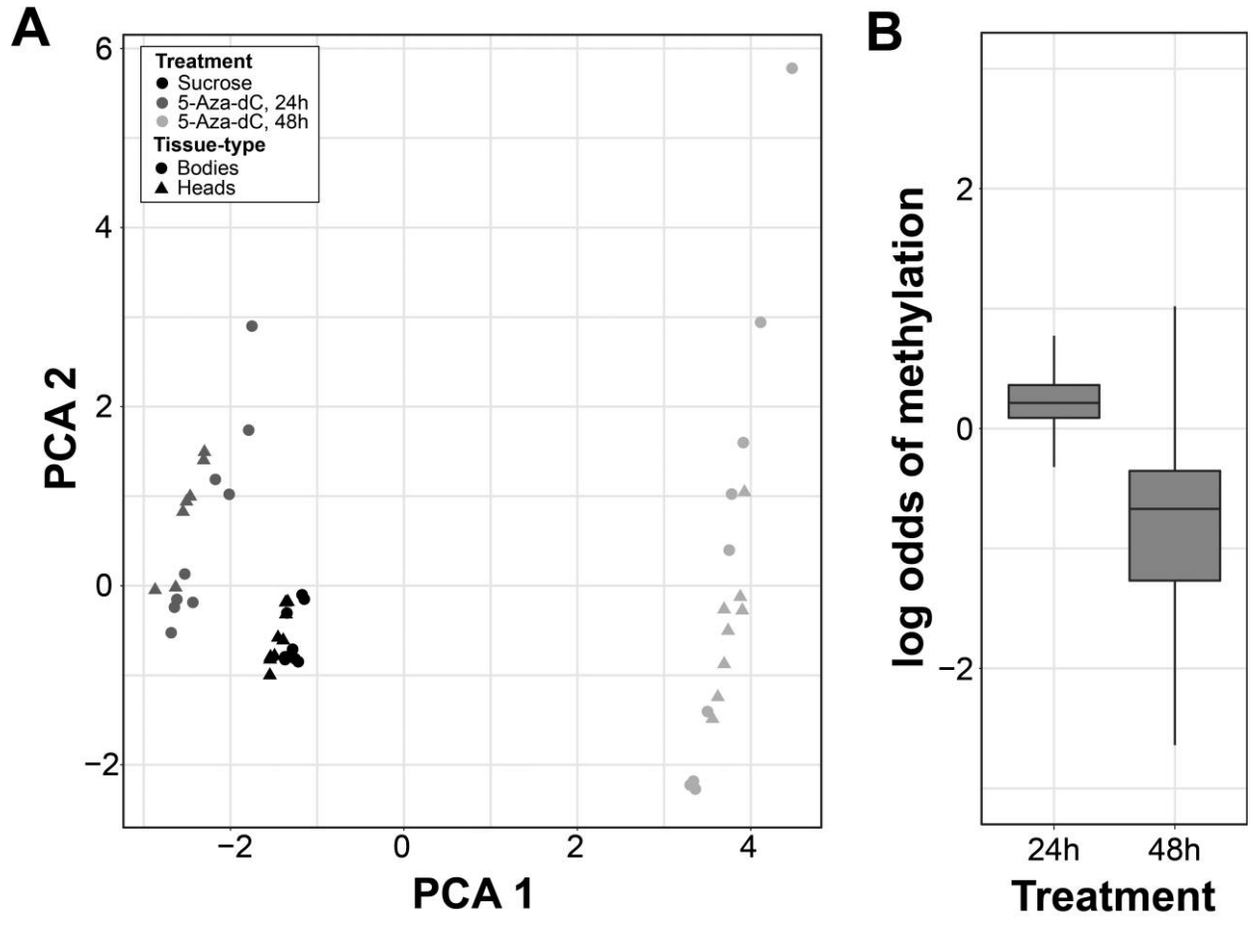

Figure 2: Based on data from Cook et al. (2018). *A*, Principal component analysis (PCA) of the proportion of methylated reads per gene ($n = 11{,}582$), showing the effects of exposure regime and tissue type. Samples cluster by exposure regime with no obvious effect of tissue type. *B*, Log odds of methylation for each of the two 5-aza-2′-deoxycytidine (5-aza-dC) exposure regimes (24- or 48-h exposure period) versus the sucrose control treatment group ($n = 8{,}556$ genes).

intermethylated sites in honey bees, Amarasinghe et al. (2014) observed hypomethylation in 10 and hypermethylation in nine of 62 loci in 5-aza-dC-exposed individuals relative to controls. Also, it looks as though Ellers et al. (2019), using bisulphite amplicon sequencing, may have uncovered some CpGs that exhibit hypermethylation in 5-aza-dC-treated samples, particularly in *perq* (Ellers et al. 2019, fig. 3). It is also noteworthy that two of the nine genes reported by Ellers et al. (2019, table 3 and fig. 3) show marginally significant treatment × site interaction (*fabd*, $P = .055$; *fasn1*, $P = .079$). This reflects the fact that 5-aza-dC affects CpG sites in a context-specific manner (and possibly in different directions), an effect that is likely masked in their data by other sites that do not differ. The mechanism by which hypermethylation is brought about by 5-aza-dC is as yet unclear. Current understanding of the DNA methylation machinery suggests that there are a range of Dnmt-including complexes and that there is cross talk between them in terms of their roles in de novo and maintenance methylation (Hervouet et al. 2018). It is possible that drug-induced demethylation in one region of the genome may have altered the regulation of the DNA methylation machinery itself, such that hypermethylation occurs. Nonetheless, it is clear that 5-aza-dC alters methylation patterns in the *N. vitripennis* genome and that this is associated with the allocation of offspring sex ratio (Cook et al. 2015, 2018).

Possible Confounding Effects

Ellers et al. (2019) raised the valid concern that the antimetabolic activity of 5-aza-dC could result in a decline in the physical condition of *Nasonia vitripennis* females, indirectly producing observed phenotypic differences through a mechanism independent of DNA demethylation. While we have shown that 5-aza-dC at the dosage used in our original experiment (Cook et al. 2015) produces altered methylation patterns (see above; Cook et al. 2018), the possibility of confounding side effects should not be ignored. We do note, however, that an effect of condition-dependent sex allocation has yet to be elucidated in *N. vitripennis*, although pathological side effects of any given chemical exposure cannot be discounted. We also note that sublethal exposure to neurotoxic neonicotinoid pesticides does also influence sex allocation, albeit in the direction opposite to that seen with 5-aza-dC (i.e., more female-biased sex ratios are seen than expected; Whitehorn et al. 2015; Cook et al. 2016).

In humans, the major side effect of 5-aza-dC treatment is myelosuppression (Karahoca and Momparler 2013), a condition in which bone marrow activity is decreased, resulting in fewer red and white blood cells and platelets. It is difficult, of course, to extrapolate what the major side effect of the drug in insects might be, if any. In mammals, high-dose schedules result in an increased presence of DNA-Dnmt1 adducts, which impede DNA polymerase function, leading to growth arrest and apoptosis. However, low-dose schedules are known to capitalize on the hypomethylating capability of the drug and minimize these additional cytotoxic effects (Jones and Taylor 1980; Jüttermann et al. 1994; Issa et al. 2004; Yang et al. 2006). In trialing the cancer drug 5-aza-dC as a tool for molecular ecology and evolution studies, researchers have also used low doses to achieve DNA demethylation (Uçkan et al. 2007; Amarasinghe et al. 2014; Cook et al. 2015; Pegoraro et al. 2016; Sak 2017). In Amarasinghe et al.'s (2014) study, examining DNA methylation differences between different reproductive worker castes in *Bombus terrestris*, they used 5-aza-dC (<10 μM) on both callows (bees <1 day old) and adults (bees >1 day old). This is less than half of the lowest concentration (25 μM) used to test for genotoxic effects of 5-aza-dC in *Drosophila melanogaster* at a range of concentrations (Cunha et al. 2002). Their experimentation showed no effect of 5-aza-dC on the adult bees in contrast to the callows, which developed larger ovaries, became more aggressive, and showed altered methylation patterns (at 19 of 62 tested loci) relative to controls of the same age. The authors hypothesized that cells in adult bees may be largely postmitotic, thus decreasing the efficacy of 5-aza-dC in adults versus callows. Importantly, neither callows nor adults showed reduced activity or reproduction; indeed, callows became more reproductive. If the effects of 5-aza-dC were merely toxic, it is reasonable to assume that reduced fitness would be observed in both age groups even if altered methylation patterns occurred only in callow bees.

Ellers and colleagues quantified the lipid reserves of both control wasps and those treated with 5-aza-dC as a general measure of body condition. The authors showed that lipid reserves were significantly lower as a result of 5-aza-dC treatment and that reserves in both treatment and control groups declined over the duration of the experiment. The authors conclude that these differences in lipid reserves can be attributed only to a detrimental effect of 5-aza-dC on wasp health given that they observed no alterations in DNA methylation in a total of six genes involved in fatty acid metabolism. Given demonstrations that 5-aza-dC does produce altered methylation patterns (Amarasinghe et al. 2014; Pegoraro et al. 2016; Cook et al. 2018), it is possible that hypo- or hypermethylation in genes untested in their experiment could have induced a decrease in lipid reserves; the six genes tested cannot be regarded as representative of the entire fatty acid metabolism pathway. Interestingly, Amarasinghe et al. (2014) noted increased reproductive capability as a result of 5-aza-dC, so it is possible that the observed reduction in lipid reserves is due to the diversion of resources elsewhere. It is also unclear whether the decline in lipid reserves was sufficient to produce a true reduction in fitness in terms of offspring production or longevity, as this information is not available.

5-aza-dC and Evolutionary Ecology

Research has determined that 5-aza-dC acts to alter methylation patterns in insects and has assisted evolutionary biologists in elucidating a role for epigenetics in a range of phenotypic traits, including sex allocation, worker reproduction, and diapause (Amarasinghe et al. 2014; Pegoraro et al. 2016; Cook et al. 2018). However, a greater understanding of how 5-aza-dC brings about altered methylation could improve the precision of its use in evolutionary ecology studies. As a starting point, careful selection of the life stage of experimental individuals can prove important. The 5-aza-dC had no demonstrable effect on DNA methylation when administered to bees more than 1 day old but resulted in significant hypo- and hypermethylation in younger bees (Amarasinghe et al. 2014). This may be due to the passive mode of action of 5-aza-dC and a higher percentage of cells in adult bees being postmitotic. This effect is also likely to be dose dependent and species specific. Therefore, if no effect of 5-aza-dC is initially observed in a given species, it is worthwhile investigating the efficacy at different life stages.

On introduction to the cell, 5-aza-dC is converted to 5-aza-dCTP, but in addition, 5-aza-dC is also subject to deamination, resulting in an inactive form of the drug being produced alongside the active form. This is due to very high levels of cytidine deaminase in the human liver and spleen (Chabot et al. 1983; Momparler et al. 1985). Ellers et al. (2019) called attention to the fact that cytidine deaminase is also present in insects. Indeed, there is a cytidine deaminase–like gene in the *Nasonia vitripennis* genome (LOC107980937), which may act to degrade some of the introduced 5-aza-dC to its inactive form. To counteract this effect, the use of an inhibitor of cytidine deaminase, such as tetrahydrouridine or zebularine, has been suggested (Eliopoulos 1998). When administered in combination with 5-aza-dC in preclinical trials, the cytidine deaminase inhibitor zebularine was shown to increase the clinical efficacy of 5-aza-dC in cell lines by reducing the degradation of 5-aza-dC (Lemaire et al. 2009). Pilot experiments to determine whether the use of a cytidine deaminase inhibitor increases the efficacy of 5-aza-dC in insect study systems would be beneficial.

Concluding Remarks

Here we have discussed the mechanisms by which the demethylating agent 5-aza-dC exerts its effect and illustrated that a whole-genome approach is required to determine the efficacy of this agent in *Nasonia vitripennis*. Having used drug-induced demethylation to validate a role for epigenetic regulation in sex allocation (Cook et al. 2015, 2018), the desirable next step would be to ascertain which CpGs are involved in the expression of the sex allocation phenotype (i.e., the causal CpGs). As outlined above, research has shown that drug-induced demethylation is nonrandom and reproducible. Experimentation is warranted to determine the reproducibility and the genomic context underlying the methylation patterns produced by 5-aza-dC in *N. vitripennis* and other insect systems.

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