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DNA Methylation and Sex Allocation in the Parasitoid Wasp *Nasonia vitripennis*

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abstract: The role of epigenetics in the control and evolution of behavior is being increasingly recognized. Here we test whether DNA methylation influences patterns of adaptive sex allocation in the parasitoid wasp *Nasonia vitripennis*. Female *N. vitripennis* allocate offspring sex broadly in line with local mate competition (LMC) theory. However, recent theory has highlighted how genomic conflict may influence sex allocation under LMC, conflict that requires parent-of-origin information to be retained by alleles through some form of epigenetic signal. We manipulated whole-genome DNA methylation in *N. vitripennis* females using the hypomethylating agent 5-aza-2′-deoxycytidine. Across two replicated experiments, we show that disruption of DNA methylation does not ablate the facultative sex allocation response of females, as sex ratios still vary with cofoundress number as in the classical theory. However, sex ratios are generally shifted upward when DNA methylation is disrupted. Our data are consistent with predictions from genomic conflict over sex allocation theory and suggest that sex ratios may be closer to the optimum for maternally inherited alleles.

Keywords: DNA methylation, sex ratio, local mate competition, genomic conflict, epigenetics, haplodiploidy.

Introduction

The role of epigenetic mechanisms in the regulation and evolution of behavior is becoming increasingly recognized. Changes in the epigenetic status of DNA provide mechanisms for the control of gene expression, with processes such as DNA methylation being associated with the upregulation or downregulation of genes that influence behavior. Some of the clearest examples to date come from the growing body of evidence that epigenetic state is associated with differences in behavior among members of social insect colonies (e.g., Kucharski et al. 2008; Lyko et al. 2010; Smith et al. 2012; Amarasinghe et al. 2014). However, epigenetic processes may also be associated with genomic imprinting (Haig 2002; Patten et al. 2014). Genomic imprinting is the epigenetic phenomenon by which alleles in offspring vary in their pattern of expression as a result of the parent from which those alleles are inherited. This means that a maternally inherited allele (inherited from the mother and called a matrigene by Patten et al. 2014) has a different pattern of expression from a paternally inherited allele (inherited from the father; a patrigene), leading to parent-of-origin specific gene expression (POGE).

Much of the interest in terms of the evolution of genomic imprinting has revolved around intragenomic conflict, whereby different actors—such as males and females or parents and offspring—are under opposing forces of selection to control gene expression in focal individuals, which might include themselves (e.g., Spencer 2000; Haig 2002; Úbeda and Haig 2004; Rice 2013; Holman and Kokko 2014; Patten et al. 2014). Under the kinship theory of genomic imprinting, the conflicts arise due to differences among individuals in their patterns of relatedness to each other; for instance, females are always related to all of their offspring, but males may be related to only some of the offspring of a female he has mated with. This means that males would be favored by selection if they could manipulate their offspring to obtain as many resources as possible from their mother. This can bring maternally and paternally inherited alleles within an individual into conflict, for instance, over maternal investment, and POGE is expected to evolve (Haig 2002). Epigenetic mechanisms then provide the opportunity to influence the expression of particular alleles. DNA methylation is a key molecular mechanism of imprinting, whereby differing "imprints" are laid down in eggs and sperm, and the subsequent inheritance of these marks leads to differential gene expression at imprinted loci in the offspring (Reik and Walter 2001).

Here, we investigate the role of DNA methylation in facultative sex allocation behavior in the parasitoid wasp *Nasonia vitripennis* and what this role could mean for

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intragenomic conflict over sex allocation. Female *Nasonia* allocate sex broadly in line with Hamilton's theory of local mate competition (LMC; Hamilton 1967, 1979; Burton-Chellew et al. 2008; West 2009). As with all Hymenoptera, *N. vitripennis* is haplodiploid, with males developing from unfertilized (haploid) eggs, and females developing from fertilized (diploid) eggs. This means that females are in putative control of sex allocation, either releasing sperm stored in their spermatheca to fertilize an egg prior to oviposition, hence producing a daughter, or not releasing sperm and instead laying an unfertilized egg that will develop into a son. Females laying eggs alone on their blowfly pupae hosts produce very female-biased sex ratios (considered throughout as proportion male). As mating takes place when adults emerge from the remains of the host (males are brachypterous in this species, with adult females dispersing away after mating), this female bias reduces competition among sons for mates and maximizes the number of mates for those sons (Taylor 1981). However, females produce less-biased, or even male-biased, sex ratios, depending on the number of other females (foundresses) ovipositing together on a host and the relative clutch sizes produced by those females (Hamilton 1967; Werren 1980; West 2009). While we have a detailed understanding of the information used by female *Nasonia* during sex allocation (e.g., Werren 1980, 1983; King et al. 1995; Shuker and West 2004; Shuker et al. 2005, 2006*a*; Burton-Chellew et al. 2008), we have only a rudimentary understanding of the genetic basis of sex allocation (Pannebakker et al. 2008, 2011, 2013).

Recent theory has shown that maternally and paternally inherited alleles have different patterns of optimal sex allocation under LMC (Wild and West 2009). In haplodiploids such as *Nasonia*, maternally inherited alleles in a female are more closely related to female offspring than paternally inherited alleles. This means that maternally inherited alleles are selected to produce more female-biased sex ratios than their paternally inherited counterparts (fig. 1*A*), although the predicted differences in species with female control of sex allocation (as in *Nasonia*; but see Shuker et al. 2006*b*) are small. Crucially, parent-of-origin information must be associated with each allele, for instance, via epigenetic marks (see "Discussion" for further information).

Recent work has confirmed genome-wide DNA methylation in *Nasonia*, with approximately 30% of genes having methylated CpG sequences, mostly in exons, with methylation typically associated with increased gene expression (Wang et al. 2013; see also Beeler et al. 2014). Here we use the demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) to manipulate the genome-wide DNA methylation status of female *N. vitripennis*. There are a number of possible outcomes of this manipulation. First, the sex allocation machinery, from the perception of LMC cues (e.g., Shuker and West 2004) to the control of fertilization, could be independent of any influence of DNA methylation. We have recently shown that facultative sex allocation in *N. vitripennis* is not associated with changes in gene expression, even though oviposition itself is (Pannebakker et al. 2013; N. Cook, U. Trivedi, B. Pannebakker, M. Blaxter, M. Ritchie, E. Tauber, T. Sneddon, and D. Shuker, unpublished manuscript), so there may be limited scope for DNA methylation to influence short-term gene expression of sex ratio–related genes. On the other hand, disrupting DNA methylation could destroy facultative sex allocation, with

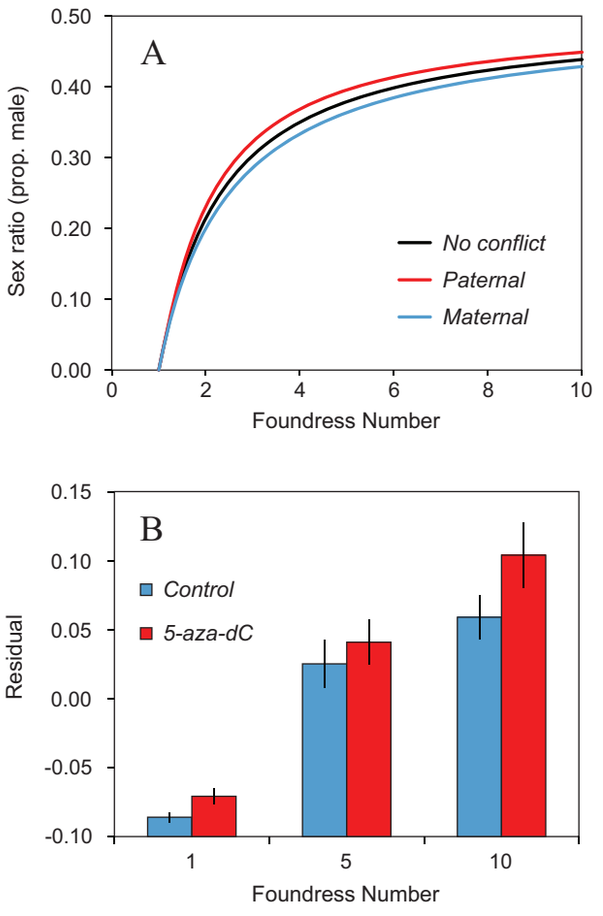

Figure 1: Sex allocation and genomic conflict. *A*, The optimal sex ratios under local mate competition for maternally inherited alleles (blue curve) and paternally inherited alleles (red curve) for a haplodiploid with female control of sex allocation (the black curve is the local mate competition prediction with no genomic conflict; equations given in Wild and West 2009). *B*, Females treated with 5-aza-2′-deoxycytidine (red bars) produce slightly less female-biased sex ratios than controls (blue bars). Both treated and untreated females vary sex ratio with foundress number. Data are presented as residuals after controlling for experimental replicate. Error bars are binomial confidence intervals.

females producing either (i) effectively random sex ratios (mean of 0.5, high variance), (ii) pathologically low sex ratios (all or nearly all daughters), or (iii) pathologically high sex ratios (all or nearly all sons). In the case of (ii), this would suggest that females might be expected to produce sex ratios similar to that of single foundresses (in the range of 0.1–0.2) regardless of the numbers of foundresses on a patch and the level of LMC that male offspring will experience. This might suggest that disrupting DNA methylation is disrupting information gathering and/or processing. In the case of (iii), this might suggest that disrupted methylation leads to disruption of control over fertilization, with females no longer able to fertilize eggs (thereby producing only sons). The remaining alternative is that facultative sex allocation is not destroyed, and females still shift sex ratios with increasing foundress numbers but the shape of the LMC curve is altered (Wild and West 2009; fig. 1*A*). We do not have an a priori prediction in this instance, as the shift in the LMC curve will depend on whether what we usually see (and in our control females) is closer to a maternally inherited allele optimum, a paternally inherited allele optimum, or a form of evolutionary compromise between the two. However, if the genes of interest follow the pattern of being upregulated when methylated (Wang et al. 2013), then an overall increase in sex ratio across the LMC reaction norm following our demethylation manipulation would imply the silencing of genes associated with producing a lower sex ratio.

Material and Methods

Nasonia vitripennis are gregarious parasitoids of dipteran pupae (Whiting 1967). The focal females used in this experiment were from the wild-type AsymC strain, the genome of which was recently sequenced (Werren et al. 2010). Wasps are maintained on *Calliphora* spp. hosts at 25°C with a 16L:8D photoperiod. Cofoundresses were drawn from the red-eye mutant *STDR* strain, allowing us to track the offspring of a focal AsymC female using eye color.

We used 5-aza-dC (Sigma Aldrich, Gillingham, UK) at a nonlethal concentration to reduce methylation across the genome in adult female *N. vitripennis*. Widely used in epigenetic anticancer treatments (Jones and Taylor 1980; Seidel et al. 2012; Yang et al. 2014), 5-aza-dC is a hypomethylating agent that works by inhibiting DNA methyltransferase activity (Creusot et al. 1982; Christman 2002). During continued exposure, 5-aza-dC molecules are incorporated into DNA during replication in the place of cytosine. This results in a passive loss of methylation across the genome as DNA methyltransferases become irreversibly bound to the 5-aza-dC residues in DNA, thereby inhibiting their function.

Mated AsymC females were collected from stock populations and individually allowed to parasitize three *Calliphora vicina* hosts. These hosts were incubated, and emerging brothers and sisters were allowed to mate for 48 h. We then took one female per sibship and individually pretreated each female with access to a fresh host for 24 h (which allows host feeding and egg development). We then allocated experimental females to one of two treatments: (1) control females, which were fed a 20% sucrose solution from a microcentrifuge tube lid for 48 h; and (2) treated females, which were fed a 20% sucrose solution, supplemented with 10 μM 5-aza-dC, also for 48 h. Fresh solution was provided after 24 h, and all solutions were colored with green food coloring. Subsequent dissection of 20 females from each treatment showed that all females had traces of green in the gut and, as such, were confirmed to have fed.

Treated and control females were then individually placed in one of three foundress conditions (alone, 5 foundresses, 10 foundresses) in a standard LMC manipulation (e.g., Werren 1983; Shuker and West 2004). The 5 and 10 foundress treatments consisted of a focal female and 4 or 9 *STDR* cofoundresses, respectively. In all treatment combinations, three hosts were provided. After 60 min, one-way escape tubes were fitted to the vials to allow females to disperse away from patches following oviposition if they chose to. After 24 h, we removed all females and incubated the hosts. Throughout, all wasps and parasitized hosts were kept at 25°C with a 16L:8D photoperiod. All emerging offspring were sexed, genotyped by eye color (black or red), and counted to determine the sex ratio produced by each focal female. Any clutches composed entirely of male offspring were removed from further analysis, as these are likely to have arisen from virgin females.

Given the small effect sizes predicted (Wild and West 2009) and observed (see below), the entire experiment was performed twice (with experimental block included in the analysis). A total of 520 sex ratios across two experimental blocks, with $N = 64–107$ for each treatment combination, were analyzed (raw data are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.15nj0 [Cook et al. 2015]). As is standard for sex ratio analyses (Wilson and Hardy 2002), we used a generalized linear modeling approach implemented in SPSS 21 (IBM, Armonk, NY), analyzing sex ratios with a binomial error structure and a logit link function. We used F tests to test for significance to account for slight overdispersion.

Results

As expected, untreated females produced the classic LMC response, with sex ratio increasing with foundress number (fig. 1*B*). Treated females also displayed facultative sex allocation, again producing less female-biased sex ratios with increasing foundress number (foundress number:

$F_{1,515} = 94.18$, $P < .001$; foundress number^2: $F_{1,515} = 36.49$, $P < .001$; fig. 1*B*). However, treated females produced slightly less female-biased sex ratios than control females ($F_{1,515} = 5.28$, $P = .022$), a pattern that was similar across foundresses (interactions: $P > .05$). Importantly, clutch size varied with foundress number as expected, with focal females in multifoundress groups producing fewer offspring than single foundresses ($F_{1,516} = 467.28$, $P < .0001$), but there was no difference in the clutch sizes produced by treated versus untreated females ($F_{1,516} = 0.47$, $P = .50$). This suggests that the chemical treatment of mothers did not lead to differential mortality of male or female offspring.

Discussion

Experimental manipulation of genome-wide DNA methylation using a common demethylating agent leads to subtle changes in sex allocation in *Nasonia vitripennis*. Importantly, the general shape of facultative sex allocation is not altered. Females still respond to increasing numbers of cofoundresses, as predicted by classical LMC theory (Hamilton 1967; fig. 1). This suggests that the presence or absence of epigenetic marks on genes associated with sex allocation does not influence whether facultative sex allocation occurs. However, the curve is subtly shifted up following treatment with the demethylating agent 5-aza-dC. Clutch size did not vary with 5-aza-dC treatment. Therefore, there is no evidence that embryonic lethality as a result of reduced DNA methylation in focal females (Zwier et al. 2012) has influenced the sex ratios observed.

Our data are consistent with predictions of genomic conflict over sex allocation under LMC (Wild and West 2009), whereby disrupting DNA methylation has shifted the balance between maternally and paternally inherited alleles within ovipositing female wasps. In their 2009 publication, Wild and West comprehensively examined the extent to which conflict between maternally and paternally inherited alleles could arise over the allocation of resources to male and female offspring. They explored conflict in a range of situations encompassing competitive and cooperative interactions between relatives, ploidy, and whether sex allocation is controlled by the mother or the offspring. In a haplodiploid species with maternal control over the sex ratio, it is predicted that paternally inherited alleles will favor a more even sex ratio, whereas maternally inherited alleles will favor a more female-biased sex ratio (fig. 1*A*). To understand the differing perspectives, it is necessary to consider three generations. Briefly, consider a grandmaternal allele: this allele can find its way into granddaughters via both sons and daughters. The grandpaternal allele can achieve the same only via daughters. This means that the maternally inherited allele is more closely related to daughters than the paternally inherited allele. Therefore, the maternally inherited allele places a greater relative genetic value on daughters than does the paternally inherited allele, leading to a more female-biased sex ratio. The sex ratios produced by untreated females in our experiment would appear to be closer to a maternally inherited allele optimum, with the treatment shifting sex ratios toward greater male production (as predicted for paternally inherited alleles).

Taking into consideration that methylation is typically associated with increased gene expression in *N. vitripennis* (Wang et al. 2013), we hypothesize that maternally inherited alleles might be "winning" the conflict, with methylation perhaps associated with upregulation of the maternal allele(s). This is the first (albeit indirect) experimental evidence for such a genomic conflict over sex allocation, using recently developed theory to guide our interpretation of the phenotypic changes. Of course, a more complete test requires proof of genomic imprinting, such that DNA methylation "imprints" carry parent-of-origin information across generations, for instance, by manipulating the DNA methylation status of males and females across generations using the techniques described here and observing changes in sex ratio.

Given that the *N. vitripennis* methylome has recently been sequenced (Wang et al. 2013; Beeler et al. 2014), future research will determine which methylated DNA sequences are involved in sex allocation. As we have recently shown that there is (so far) no detectable differential gene expression associated with facultative sex allocation in *Nasonia* (N. Cook, U. Trivedi, B. Pannebakker, M. Blaxter, M. Ritchie, E. Tauber, T. Sneddon, and D. Shuker, unpublished manuscript), it would appear that disrupting DNA methylation is not having its effect by changing patterns of gene expression during oviposition, but rather the effect must be upstream, perhaps changing the sex allocation machinery prior to the female actually having to allocate sex. We are still some way off of uncovering what this machinery is. More generally, our data show how elucidating the mechanism of familiar behaviors can enrich our understanding of them, in this case, showing that we may need to consider genomic conflict in our models of sex allocation under LMC. Furthermore, our data confirm that new theoretical and empirical insights are still there to be discovered, even in well-studied systems such as sex allocation in parasitoid wasps.

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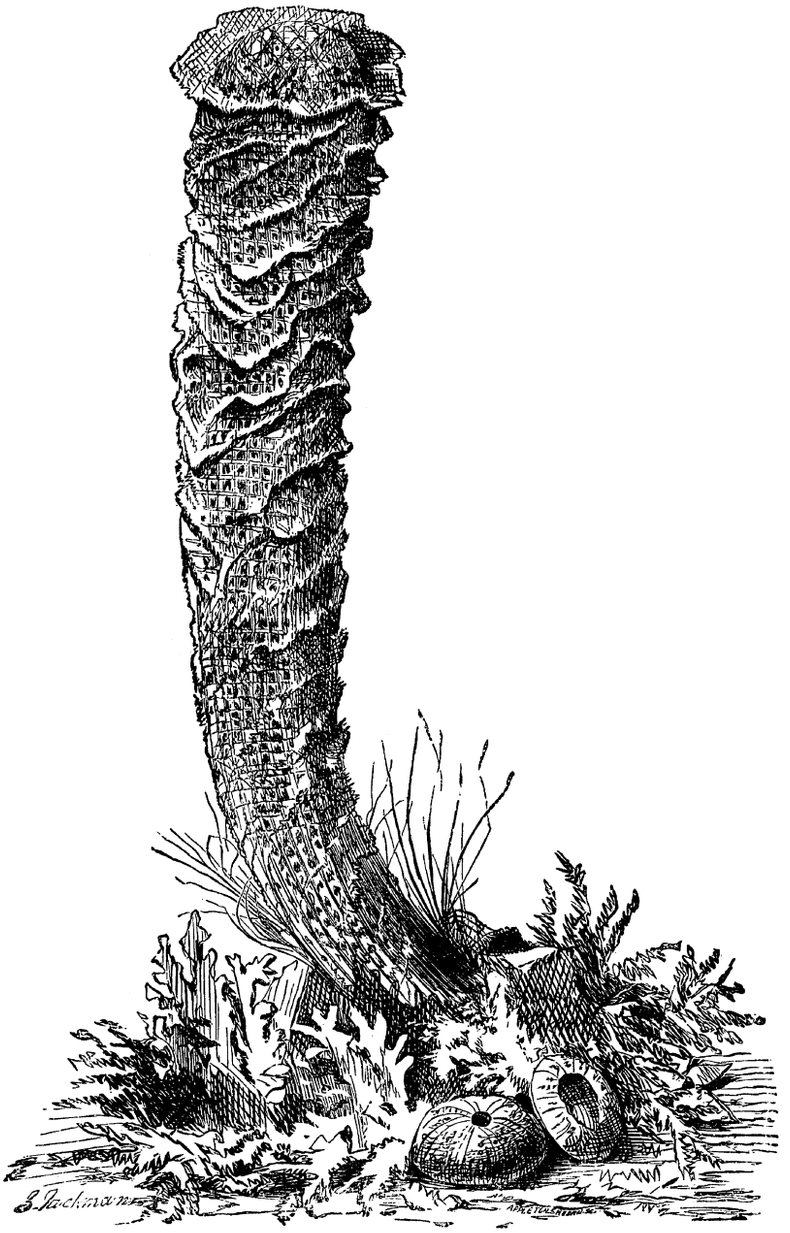

"One of the rarest, and I may say most beautiful of the silicious sponges, is the *Euplectella speciosa* Gray.... The first specimen of this remarkable sponge was purchased by Mr. Cuming, the celebrated conchologist, at the death of Mr. William J. Broderip, who had formerly given the sum of £30 to become the possessor of this then unique Euplectella." From "Sponges" by Bryce M. Wright Jr. (*The American Naturalist*, 1869, 3:449–455).