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DNA methylation plays a crucial role during early *Nasonia* development

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Abstract

Although the role of DNA methylation in insect development is still poorly understood, the number and role of DNA methyltransferases in insects vary strongly between species. DNA methylation appears to be widely present among the social hymenoptera and functional studies in *Apis* have suggested a crucial role for *de novo* methylation in a wide variety of developmental processes. The sequencing of three parasitoid *Nasonia* genomes revealed the presence of three *Dnmt1* (*Dnmt1a*, *Dnmt1b* and *Dnmt1c*) genes and one *Dnmt2* and *Dnmt3* gene, suggesting a role of DNA methylation in *Nasonia* development. In the present study we show that in *Nasonia vitripennis* all *Dnmt1* messenger RNAs (mRNAs) and *Dnmt3* mRNA are maternally provided to the embryo and, of these, *Dnmt1a* is essential during early embryogenesis. Lowering of maternal *Dnmt1a* mRNA results in embryonic lethality during the onset of gastrulation. This dependence on maternal *Dnmt1a* during embryogenesis in an organismal group outside the vertebrates, suggests evolutionary conservation of the function of *Dnmt1* during embryogenesis.

Keywords: DNA methyltransferase, Dnmt, DNA methylation, epigenetics, embryogenesis, development, maternal effect, RNAi, *Nasonia vitripennis*, insect.

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Introduction

DNA methylation is the covalent addition of a methyl group to predominantly CpG dinucleotides, and is mediated by DNA methyltransferases. In vertebrates, DNA methylation represents one of the key epigenetic modifications that have been associated with the regulation of gene expression and chromatin structure, in particular during early development (Geiman & Muegge, 2010). This process is involved in many regulatory functions, including X-chromosome inactivation, DNA repair and stability, cell differentiation, alternative splicing, and the establishment of parent-of-origin-specific gene expression (Schwartz & Ast, 2010). In mammals, differentially methylated clusters are established during gametogenesis by the '*de novo*' methyltransferase DNMT3, while imprinting is maintained by the methyltransferase DNMT1 (Hermann *et al*., 2004). The DNMT2 methyltransferase is mainly involved in tRNA methylation (Goll *et al*., 2006).

In contrast to vertebrates, *Drosophila melanogaster* lacks both *Dnmt1* and *Dnmt3* genes and, accordingly, has a sparingly methylated genome without a clearly defined functional significance of methylated sites. Initially, this called into question whether there was any evolutionary or functional role of DNA methylation in insects (Mandrioli & Borsatti, 2006; Phalke *et al*., 2009; Krauss & Reuter, 2011); however, the discovery of a functional methylation system in the honeybee, consisting of two *Dnmt1* genes and one copy each of *Dnmt2* and *Dnmt3*, provided the first indication for the existence of both *de novo* and maintenance methylation in insects (Wang *et al*., 2006). Subsequently, an increasing number of insect genomes have been sequenced, showing a large diversity in the number of *Dnmt* genes present (Lyko & Maleszka, 2011). DNA

methylation genes have been found among multiple species of wasps, bees and ants representing the social hymenoptera, although functional studies have been largely confined to the honeybee (Kronforst *et al*., 2008). Methylation in adult honeybees (*Apis mellifera*) seems to be restricted to CpG dinucleotides and occurs mainly in gene bodies, particularly in the proximity of alternatively spliced exons, suggesting a role in splicing regulation (Lyko *et al*., 2010). Kucharski *et al*. (2008) showed that siRNA-mediated silencing of the expression of *Dnmt3* in newly hatched honeybee larvae gave rise to the development of adult worker bees with developed ovaries, which is normally associated with queen development only. Further roles for *Dnmt3* in the honeybee have been shown in the regulation of behaviour and long-term memory (Maleszka *et al*., 2009; Lockett *et al*., 2010). In addition, the observed lethality associated with *Dnmt3* silencing in embryos suggests a crucial role during embryonic development (Kucharski *et al*., 2008). Altogether, these results indicate that *de novo* DNA methylation is crucial for a wide variety of developmental processes in *Apis*, and it is of interest whether this feature is general throughout the hymenopteran insects.

The *Nasonia* species group has emerged as a new important hymenopteran model system for investigating complex life history traits and development. The sequencing of the *Nasonia* genome revealed three *Dnmt1* genes (*Dnmt1a*, *Dnmt1b* and *Dnmt1c*), one *Dnmt2* gene and one *Dnmt3* gene. *Dnmt1a* and *Dnmt1b* are structurally very similar, while *Dnmt1c* is less similar to both *Nasonia Dnmt1a* and *Dnmt1b*, but has the invariant cysteine required for catalytic activity (Werren *et al*., 2010). Of the three *Dnmt1* homologues present on the *Nasonia* genome, *Dnmt1a* also shows sequence similarity to *Dnmt1* homologues in other species, including humans (Werren *et al*., 2010). This, in conjunction with the fact that all three *Dnmt* genes were present in the last common ancestor of both invertebrates and vertebrates, suggests an ancestrally conserved function for DNA methylation (Werren *et al*., 2010; Lyko & Maleszka, 2011). Moreover, despite the broad diversity of sequences shown to be methylated, and the complex, dynamic patterns in which they occur on the vertebrate genome, gene body methylation is suggested to represent an ancient property of the eukaryotic genome (Zemach *et al*., 2010).

Being a hymenopteran species, *Nasonia* has haplodiploid sex determination, in which males emerge from haploid unfertilized eggs and females are diploid. Recently, it was shown that *Nasonia* sex determination involves maternal provision of *transformer* (*Nvtra*) messenger RNA (mRNA) to the eggs, combined with parental imprinting to regulate zygotic *transformer* (*Nvtra*) expression (Verhulst *et al*., 2010). In addition, Park *et al*. (2011) showed distinct patterns of methylated genes in the genome of *Nasonia*, among them the *Nvtra* gene, which may function as regulators of gene function. During embryonic development in *Nasonia*, the early pronucleus initially goes through several rounds of rapid mitoses without cytokinesis to form the syncytium (Bull, 1982; Pultz & Leaf, 2003; Pultz *et al*., 2005), followed by the first onset of zygotic transcription 5 h after egg laying (at 25°C) (Verhulst *et al*., 2010). Conservation of DNA methylation patterns during these early mitoses would therefore suggest maternal provision of *Dnmt* mRNA. To evaluate this, we examined the effect of parental RNA interference (pRNAi) of *Dnmt* gene expression in the present study. The results show an important role for maternal provision of *Dnmt1a* in early embryonic development, suggesting a crucial function for DNA methylation during early *Nasonia* development.

Results

Maternal input of Dnmt *mRNA*

Quantitative real-time PCR (qPCR) analysis showed that both *Dnmt1a* and *Dnmt1c* genes are expressed in the ovaries and that the corresponding mRNAs are maternally provided to the eggs. In contrast, *Dnmt1b* appeared to be nearly absent in ovaries and early embryos (Fig. 1). The maternally provided amount of mRNA for *Dnmt1a* and *Dnmt1c* is about 3–5-fold higher than that of maternally provided *Nvtra*, that was chosen as a reference. However, the normalized expression levels of *Dnmt1a* and *Dnmt1c* do not significantly differ from one another (Kruskal–Wallis one-way ANOVA on ranks). *Dnmt3* mRNA is also maternally provided, although at lower level than *Dnmt1a* and *Dnmt1c*.

Parental RNA interference of Dnmt1c *and* Dnmt3

Because *Dnmt1c* showed the highest level of maternal input, pRNAi in the white pupal stage (Lynch & Desplan,

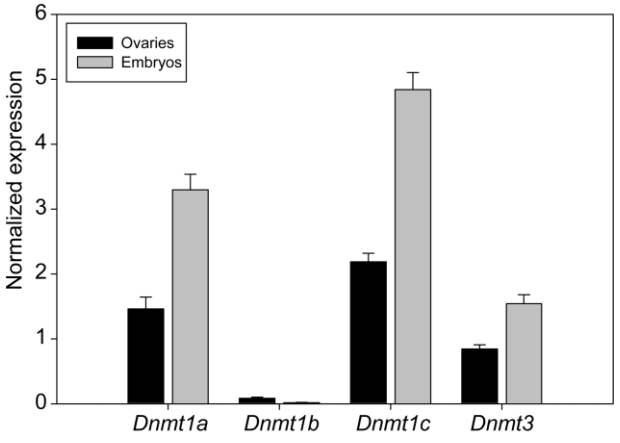

Figure 1. Relative expression levels of *DNA methyltransferases* in ovaries (■) and levels of maternal input of *DNA methyltransferases* in early embryos (□). Error bars represent SE.

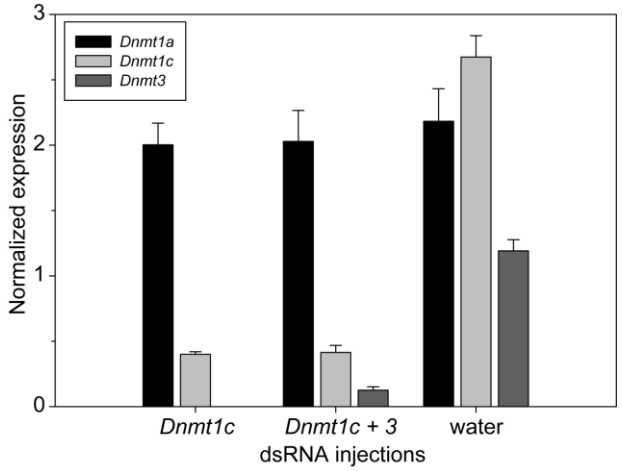

Figure 2. Relative expression levels in adult (4-day-old) females of *Dnmt1a* (■), *Dnmt1c* (□) and *Dnmt3* (▩) after parental RNAi of *Dnmt1c*, and *Dnmt1c* combined with *Dnmt3*. Sterile water was used as an injection control. Error bars represent SE.

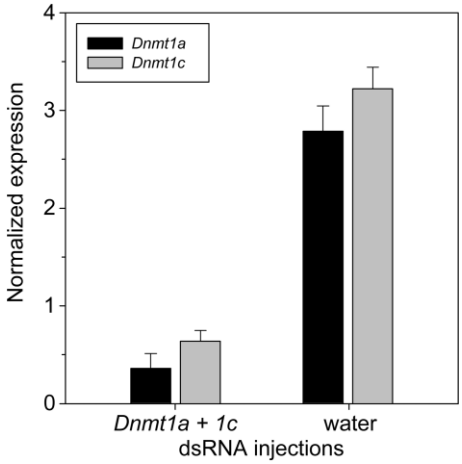

Figure 3. Relative expression levels in adult (4-day-old) females of *Dnmt1a* (■) and *Dnmt1c* (□) after parental RNAi knockdown of *Dnmt1a* combined with *Dnmt1c*. Sterile water was used as an injection control. Error bars represent SE.

2006) was applied to reduce maternal provision of *Dnmt1c* mRNA. qPCR analysis showed that *Dnmt1c* mRNA levels were significantly lowered in injected females, compared with the controls (one-tailed *t*-test, $P < 0.01$, Fig. 2). *Dnmt1c* pRNAi did not, however, lead to a decrease in *Dnmt1a* expression levels (Fig. 2), even though the target region of *Dnmt1c* shows similarity to that of *Dnmt1a*, demonstrating the specificity of the pRNAi knockdowns. After emergence, injected females showed no aberrant phenotype; they were able to mate and oviposit and produced normal clutch sizes. This result shows that expression of *Dnmt1c* is not essential for development from the white pupal stage onward. Moreover, the lowering of *Dnmt1c* maternal input had no apparent effect on the development of their offspring. Apparently, maternal provision of the maintenance DNA methyltransferase *Dnmt1c* mRNA is also not essential for embryonic development.

Combination of the pRNAi of *Dnmt1c* and *Dnmt3* showed results identical to those of *Dnmt1c* alone. Neither the development of the treated females nor the development of the offspring were affected by the injections, although the levels of both mRNAs were significantly lowered (one-tailed *t*-test, $P < 0.01$, Fig. 2). These results show that expression of *Dnmt3* is not essential from the white pupal stage onward and that maternal provision of *Dnmt3* mRNA is not essential for embryonic development.

Parental RNA interference of Dnmt1a *and* Dnmt1c

Parental RNAi of *Dnmt1a* and *Dnmt1c* combined also had no effect on the development and phenotype of the injected females. The effectiveness of pRNAi of *Dnmt1a* and *Dnmt1c* in the injected pupae was confirmed by qPCR (one tailed *t*-test, $P < 0.01$, Fig. 3). This result shows that the normal high level of expression of *Dnmt1a* is not essential for development from the white pupal stage onward. However, none of the offspring of the injected females were viable, indicating that depletion of maternally provided *Dnmt1a* and *Dnmt1c* together results in embryonic lethality. As was shown in the previous section, silencing *Dnmt1c* alone or in combination with *Dnmt3* did not result in any aberrant phenotype in the treated females or their offspring, and it was concluded, therefore, that this lethality was caused by the depletion of maternally provided *Dnmt1a* mRNA. To confirm this, the pRNAi experiment was repeated with *Dnmt1a* double-stranded (ds)RNA alone. As this resulted in the same embryonic lethality, we concluded that the successful knockdown of *Dnmt1a* expression (one-tailed *t*-test, $P < 0.01$, Fig. 4) in

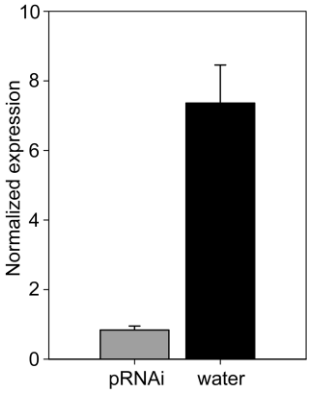

Figure 4. Relative expression levels in adult (4-day-old) females of *Dnmt1a* after *Dnmt1a* (□) parental RNA interference (pRNAi) or water-injected controls (■). Error bars represent SE.

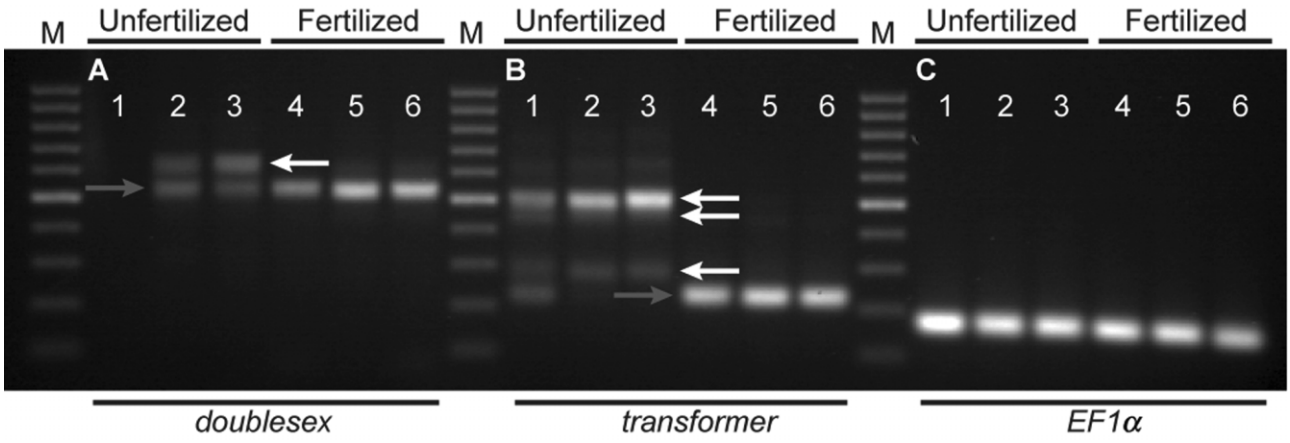

Figure 5. Splice forms of *Nasonia vitripennis doublesex* (*Nvdsx*) and *N. vitripennis transformer* (*Nvtra*) mRNA in fertilized or unfertilized 12-h-old embryos, produced by *Dnmt1a* combined with *Dnmt1c* double-stranded (ds)RNA-injected females. White arrows indicate male specific splice variants; grey arrows indicate female specific splice variants. Panel A: Sex-specific splicing of *Nvdsx* mRNA. The presence of the female specific splice form in male samples is commonly observed (Oliveira *et al*., 2009). Panel B: Sex-specific splicing of *Nvtra* mRNA. Panel C: The amplification of *EF1α* is used as a cDNA control. M denotes 100 bp molecular weight marker. The absence of any signal and the presence of the female splice form of *Nvtra* in sample 1 in panels A and B, respectively, may indicate the developmental arrest of this sample in an earlier stage (~7h).

the white pupal stage results in reduction of maternally provided *Dnmt1a* mRNA to the eggs and, subsequently, to developmental arrest and lethality. pRNAi of males in the white pupal stage did not have any effect on their further development or their offspring (data not shown). This confirms that the observed pRNAi phenotype is caused by a maternal effect.

We previously showed that maternal input of *Nvtra* mRNA in combination with epigenetic control of zygotic *tra* expression, starting 5 h after fertilization, is essential for female-specific splicing of *Nvtra* and *doublesex* (*Nvdsx*) mRNA (Verhulst *et al*., 2010). Therefore, we determined whether silencing of *Dnmt1a* and *Dnmt1c* combined had an effect on the sex-specific splicing of *Nvtra* and *Nvdsx* mRNA. The female-specific splicing of *Nvdsx* is dependent on the presence of an active NvTRA, which is produced from the female but not from the male splice form of *Nvtra*. Unfertilized haploid eggs will develop as males, while fertilized diploid eggs start the female developmental route 7 h after egg laying. Surprisingly, as is shown in Fig. 5, 12-h-old embryos produced by *Dnmt1a* and *Dnmt1c* pRNAi-treated mated and unmated females showed normal splicing of both *Nvtra* and *Nvdsx* according to their ploidy level. Apparently, even though combined *Dnmt1a* and *Dnmt1c* pRNAi treatment leads to developmental arrest from 10–12 h onwards, the sex-specific expression and splicing of both *Nvtra* and *Nvdsx* is not disturbed. These results show that the sex-specific splicing of *Nvtra* is not critically dependent on maternal provision of *Dnmt1a* or *Dnmt1c* mRNA and that the epigenetic silencing of zygotic *Nvtra* expression is either not dependent on maternal expression of *Dnmt1a* or *Dnmt1c* or is already established before the white pupal stage.

Developmental effects of parental RNA intereference of Dnmt1a

To determine the onset of the observed developmental arrest, embryos produced by virgin *Dnmt1a* dsRNA-treated females were collected over a temporal series of 1-h intervals from 1 h after egg laying until 12 h after egg laying. Blue-fluorescent 4',6-diamidino-2-phenylindole (DAPI) staining, which permeates the cell membrane and preferentially stains dsDNA, was used to monitor embryonic development. The results are presented for embryos of unmated females, although the observed embryonic lethality was similar for mated females. Embryos at similar developmental stages that were produced by females injected with sterile water served as controls. This developmental series of DAPI-stained embryos showed that both sets of embryos went through a normal developmental programme initially, forming a normal syncytium and regular cellularization. DAPI staining on embryos produced after knockdown of maternally provided *Dnmt1a* mRNA and control embryos showed that 0–1 h after egg laying (Fig. 6A, E), no mitosis had occurred in developing embryos of both groups while normal positioning of the pronucleus was observed (Bull, 1982). No effects of developmental arrest were observed at 4 h after egg laying (Fig. 6B, F), when embryos of both groups had gone through several rounds of nuclear mitosis without cytokinesis to form the syncytium (Pultz & Leaf, 2003; Pultz *et al*., 2005). The nuclear divisions appeared synchronous in both groups, pole cells migrated

normally to the posterior end of the embryo and nuclear migration to the periphery of the embryos marked the start of the blastula stage (Bull, 1982; Pultz & Leaf, 2003). The first differences were observed 10–12 h after the eggs were deposited (Fig. 6C, G), when small invaginations could be seen in nearly 100% of the control embryos, marking the start of segmentation and onset of gastrulation. These invaginations could not be observed in the embryos from the injected females. Instead, all of the embryos stopped developing at this same developmental stage.

Discussion

The identification of *Dnmt* genes in several insect species (The Honeybee Genome Sequencing Consortium, 2006; Wang *et al*., 2006; Walsh *et al*., 2010; Werren *et al*., 2010; Xiang *et al*., 2010; Zemach *et al*., 2010) has directed attention to the possible role of DNA methylation in establishing the complex insect phenotype. For several insect species, patterns of genomic methylation have been reported, indicating a relatively low percentage of methylated sites compared with vertebrates (see Lyko *et al*., 2010 and references therein), and methylation sites that are mainly located in gene bodies (Kucharski *et al*., 2008; Werren *et al*., 2010; Park *et al*., 2011). However, functional analysis of DNA methylation has thus far only been reported for the honeybee (Kucharski *et al*., 2008; Maleszka *et al*., 2009; Lockett *et al*., 2010; Lyko *et al*., 2010). In the present study, we demonstrate an essential function for maternally provided *Dnmt1a* mRNA in *Nasonia* development.

The hymenopteran *Nasonia*, like the dipterans *Drosophila* and *Musca* form so-called 'long germ' embryos. Although *Nasonia* diverged from diptera more than 200 million years ago, like *Drosophila* it starts developing in a syncytial environment in which germ patterning is strongly dependent on maternal factors (Pultz & Leaf, 2003; Rosenberg *et al*., 2009). Lynch *et al*. (2006) and Lynch & Roth (2011) showed that the *Nasonia* zygote relies heavily on maternal input of mRNA gradients for axis formation and germline determination. In addition, Verhulst *et al*. (2010) showed that maternal input of *Nvtra* mRNA, in combination with specific zygotic *Nvtra* transcription and *Nvtra* autoregulation of female-specific splicing, is essential for female development of *Nasonia* embryos, while males develop as a result of maternal imprinting that prevents zygotic *Nvtra* transcription. These combined results led to the hypothesis that maternal provision of *Dnmt* mRNA may play an important role in *Nasonia* development. Indeed, our results indicate that all *Dnmts*, except *Dnmt1b*, are maternally provided. Strikingly, only knockdown of maternally provided *Dnmt1a* mRNA resulted in an observable phenotype, where embryonic development is arrested 10–12 h after egg laying, coinciding in time with the onset of gastrulation (Bull, 1982). This finding suggests that maintenance of DNA methylation patterns is an important feature of early *Nasonia* development and is in part mediated by maternal provision of *Dnmt1a*.

Despite this pronounced effect on development, no disturbance of sex-specific splicing of either *Nvtra* or the downstream *Nvdsx* gene was observed. Since this sex-specific splicing is dependent on the zygotic expression of *Nvtra*, our results indicate that the activation and expression of zygotic *Nvtra* does not critically depend on maternally provided *Dnmts*. Moreover, pRNAi using all *Dnmt* dsRNA never led to the production of diploid males during the present study, whereas this was a crucial finding in our previous studies on sex determination using *Nvtra* dsRNA. As there was no zygotic expression of *Dnmt1* genes to maintain maternally determined methylation patterns, these observations may contradict the suggestion of Park *et al*. (2011) that the preferential methylation pattern of CpG sites of exon 2 of *Nvtra* plays a role in *Nasonia* sex determination by marking alternative splicing sites of this gene. Alternatively, the residual *Dnmt* mRNA levels after pRNAi may be sufficient to maintain this pattern. It should be noted, however, that the underlying mechanism of splicing regulation by DNA methylation is unknown (Glastad *et al*., 2011). Clearly, this issue requires more detailed research.

Another important result is our finding that maternal provision of *Dnmt3* mRNA is not essential for either early development or sex determination of the offspring, despite the fact that in the honeybee larval expression of this gene is involved in caste determination (Kucharski *et al*., 2008). Perhaps the effect of *Dnmt3* in the honeybee only becomes apparent in the larval stage and can hence be independent of maternal provision. Moreover, the function of *Dnmt3* in caste formation in *A. mellifera* is context-dependent and its absence does not necessarily reflect an abnormal developmental situation. It would be interesting to know if *Dnmt3* possesses such context-dependent function in *Nasonia* as well. In addition, in the honeybee the germline does not appear to be specified maternally (Dearden *et al*., 2006; Rosenberg *et al*., 2009), in contrast to that of *Nasonia* (Lynch *et al*., 2011). Since *Nasonia* development is heavily dependent on maternal factors, this raises the question why no apparent phenotypic effect is observed when maternal provision of *Dnmt3* and *Dnmt1c* is prevented. A possible explanation could be that these genes are less important until the onset of maternal zygotic transition (MZT). In *D. melanogaster* some 30% of maternal mRNAs degrade markedly at MZT, while others are more stable (De Renzis *et al*., 2007). Since *Nasonia* has a relatively long syncytial pre-blastoderm stage (Pultz *et al*., 1999), perhaps zygotic expression for *Dnmt1c* and *Dnmt3* is

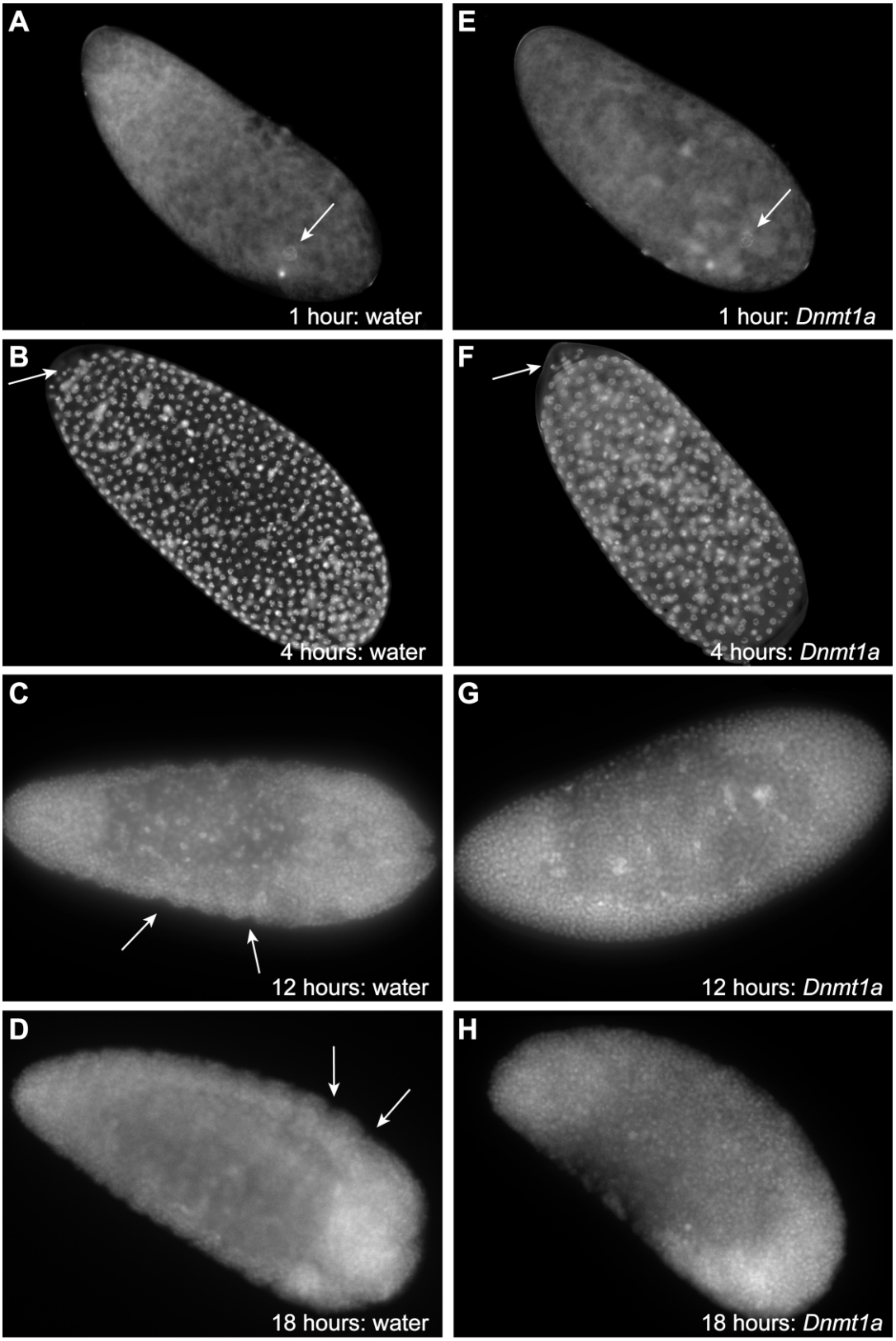

Figure 6. Blue-fluorescent 4′,6-diamidino-2-phenylindole (DAPI) staining on embryos produced by *Dnmt1a* double-stranded (ds)RNA-injected females and embryos produced by females injected with sterile water as a control. (A, E) 0–1 h after egg laying, no mitosis has occurred in the developing embryo, both groups show normal positioning of the pronucleus (⟶) (Bull, 1982). (B, F) 4 h after egg laying, embryos of both groups have gone through several rounds of nuclear mitoses without cytokinesis to form the syncytium (Pultz *et al*., 2005). The nuclear divisions appear synchronous in both the *Dnmt1a*-injected and control group. Pole cells (⟶) have normally migrated to the posterior end of the embryo in both the dsRNA- and water-injected groups. Nuclear migration to the edge of the embryos is beginning, marking the start of the blastula stage, just prior to cellularization and activation of the zygotic genome (Bull, 1982; Pultz & Leaf, 2003). (C, G) After 12 h, small invaginations can be observed in the water-injected embryos (⟶) marking the start of segmentation and onset of gastrulation. These invaginations are not observed in the *Dnmt1a* parental RNA interference (pRNAi)-treated embryos. The embryos from this group stopped developing at this time point. (D, H) After 18 h, a clear difference can be observed between the water-injected and *Dnmt1a* pRNAi embryos. Whereas the embryos from the water-injected control group have advanced well into gastrulation, no further development could be observed for the *Dnmt1a*-injected group.

early enough to overlap the maternal input and counteract the lowered levels of maternally provided mRNAs through pRNAi. We are currently exploring zygotic expression levels in combination with larval RNAi to explore these questions in more detail.

In conclusion, we have found that *Dnmt* mRNA is maternally provided to the *Nasonia* embryo and that maternally provided *Dnmt1a* mRNA is vital for early embryonic development. It is interesting to note that the necessity for *Dnmt1* during embryogenesis has been demonstrated in a number of species thus far, including mouse, frog and zebrafish. Overall, *Dnmt1* deficiency in these species has been shown to result in misexpression of genes that specify embryonic cell identity but to have limited effects on early developmental mitoses (Li *et al*., 1992; Stancheva & Meehan, 2000; Jackson-Grusby *et al*., 2001; Stancheva *et al*., 2001; Rai *et al*., 2006). The similarities in the observed effects of *Dnmt1a* knockdown in *Nasonia* and the apparent sequence conservation of *Dnmt1* might mean that some of its functions are more conserved than previously thought. The notion that DNA methylation is ancestral to insects and that the lineage-specific differences of the number of *Dnmt* genes reflect the evolutionary diversification of insects (Glastad *et al*., 2011) further suggests a dynamic pattern of the developmental roles for DNA methyltransferases in insect evolution (Lyko *et al*., 2010; Werren *et al*., 2010).

Experimental procedures

Insect strains

The *Nasonia vitripennis* wild-type laboratory strain AsymC, which is cured from *Wolbachia*, and the recessive red eye colour mutant strain st^{DR} were used throughout the experiments. All wasp culturing was done on pupae of *Calliphora vicina* flies at 25°C, unless stated otherwise.

RNA extraction and cDNA conversion

Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. All isolated total RNA was subsequently reverse transcribed according to protocol with a mix of one part oligo-dT and six parts random hexamer, both provided with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA).

Quantitative Real-Time PCR

Quantitative real-time PCR analysis was done using 2 μl of a 10-fold cDNA dilution and ABsolute™ QPCR SYBR Green ROX (500 nM) Mix (Abgene, Hamburg, Germany) on an Applied Biosystems 7300 Real Time PCR System (Foster City, CA, USA) with 300 nM qPCR primers for *Dnmt1a*, *Dnmt1b*, *Dnmt1c* and *Dnmt3* (see Table 1). The *transformer* gene expression was used as reference, using 300 nM of primers Nvtra_qPCR_F1 and Nvtra_qPCR_R1 that amplify a non-sex specific part of the transcript (Verhulst *et al*., 2010). All primers sets (Table 1) were developed to contain at least one exon–exon spanning primer using PerlPrimer (Marshall, 2004). Primer sets showed no amplification from the genomic DNA template. qPCR profile was as follows: 95°C for 15 min, 40 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s. Standard curve for 1:10.000 fold cDNA dilutions were run to determine linearity and primer efficiencies. A standard ABI7300 dissociation curve was applied to control for nonspecific amplification. LinregPCR (Ramakers *et al*., 2003) was used for calculating starting concentrations of both genes. Statistical analysis was performed using SPSS 16.

Expression analysis of Dnmt *in ovaries and early embryos by quantitative real-time PCR*

Sixty females from the st^{DR} line were individually setup as virgin and given one fly pupa every other day for a period of 3 days. Thereafter, these females were allowed to oviposit for 3 h at 25°C in an egg-laying chamber to facilitate embryo collection. Seven replicates of 50 embryos per time point were collected in 100% ethanol at 2°C in approximately 30 min and stored at −80°C until RNA extraction. Also, seven samples of three ovaries per sample of 4-day-old females were collected in 100% ethanol at 2°C and stored at −80°C until RNA extraction. Relative expression levels of *Dnmt1a*, *Dnmt1b*, *Dnmt1c* and *Dnmt3* in embryos and ovaries were calculated by comparing these starting concentrations to starting concentrations of the reference gene *Nvtra*, which was previously shown to be maternally provided (Verhulst *et al*., 2010).

Parental RNAi

Parental RNAi knockdown was induced in white female pupae from the st^{DR} line according to protocol described by Lynch and

Table 1. Overview of the primers used during this study

	Primer name	Sequence	Ta	Amplicon bp	Exon
Quantitative real-time PCR					
Dnmt1a	qPCR1a_1_Fw	TCTACCGGAGCATTAAGAGG	58	211	9
	qPCR1a_1_Rv	CTCGTCCCTGTTTACCCATAG	58	211	9/10*
Dnmt1b	DNMT1bR_Fw	CACCTTCTTCAACGTGTACTG	58	133	1/2*
	qPCR1B_Rv	TCCTTCTATGGAAGAATCATCG	58	133	2
Dnmt1c	qPCR1c_1_Fw	GAAGATGCAGTAATCGATTCCA	58	197	6
	qPCR1c_1_Rv	ACCGTACATACTTCATTACTCC	58	197	6/7*
Dnmt3	qPCR3_Fw	CCGAATATCTGAGTTGAATGC	58	192	3/4*
	qPCR3_Rv	GCAATGTATGGCTTTACCAAG	58	192	5
RNA interference					
Dnmt1a	qPCR1a_2_Fw	CTCAAACACGAAGAAGGATGAT	58	693	5/6*
	qPCR1a_1_Rv	CTCGTCCCTGTTTACCCATAG	58	693	9/10*
Dnmt1c	RNAi_DNMT1c_Fw	TGGAGTAATGAAGTATGTACGG	58	700	6/7*
	RNAi_DNMT1c_Rv	GCATTAAATCGAACGTCACCT	58	700	9
Dnmt13	MethF	GGCATTGAAAGTATGCATTCAC	58	488	2
	MethR	GATTCACCAATCCAAGAAACC	58	488	3/4*

*Represents exon–exon spanning primers.

Desplan (Lynch & Desplan, 2006). A dsRNA fragment of 693 bp was used for *Dnmt1a*, a fragment of 700 bp for *Dnmt1c* and a fragment of 488 bp for *Dnmt3*. At either the 5′ or 3′ end of the fragment a T7 promoter was placed using designed primers for all three *Dnmt*-specific dsRNAs (see Table 1 for primer sequences). These fragments were transcribed in both directions using the Megascript RNAi kit (Ambion, Austin, TX, USA) according to protocol to generate dsRNA. Approximately 75 white female st^{DR} pupae were injected with a mixture of 4 μg/μl of *Dnmt1c* dsRNA and 2 μg/μl of *Dnmt3* dsRNA mixed with 10% red food colouring dye. Another 75 white female pupae were injected in the abdomen with a mixture of 4 μg/μl of *Dnmt1a* dsRNA and 2 μg/μl of *Dnmt1c* mixed with 10% red dye. Injections were performed with Femtotips II needles (Eppendorf, Hamburg, Germany) under continuous injection flow. The female was injected until her abdomen turned clearly pink. After the injections the females and their developing F1 progeny were placed at 30°C. After emergence, females were kept virgin or were mated to AsymC males in separated tubes, after which they were given one fly host for 4consecutive days. On day 4, seven of the injected females were randomly collected for qPCR analysis to establish whether knockdown of the target genes was successful. The hosts provided on days 2 and 4 after injection were used to determine clutch size, and sex ratio.

Expression analysis

Expression levels of *Dnmt1a*, *Dnmt1b*, *Dnmt1c* and *Dnmt3* after RNAi were assessed in the abdomen of adult females 4 days after emergence i.e. 10–11 days after dsRNA injection ($n = 7$). They were compared with the *Dnmt* expression levels in the abdomens of adult females of the same age that were injected with sterile water ($n = 7$). Relative expression levels of *Dnmt1a*, *Dnmt1b*, *Dnmt1c* and *Dnmt3* in water-injected females and *Dnmt1a*, *Dnmt1c* + *Dnmt3* or *Dnmt1a* + *Dnmt1c* dsRNA-injected females and their offspring were calculated by comparing *Dnmt* starting concentrations to starting concentrations of the reference gene *Nvtra*.

DAPI staining of embryos

Embryos were collected in 1.5 ml Eppendorf tubes containing 1 ml heptane + 300 μl phosphate-buffered saline and fixed by adding a 20% formaldehyde solution to a final concentration of 5%, followed by shaking at 100 rpm for 30 min. A series of washings with methanol was used to dehydrate the embryos for storage at −20°C. After embryos of all time points were collected, the embryos were rehydrated by gradually replacing the methanol by PBT buffer. For the DAPI stain itself, one drop of vectashield mounting medium with DAPI (Vector Laboratories, Inc. Burlingame, CA, USA) was added and incubated overnight at 4°C. The embryos were then transferred to glass microscopy slides and analyzed on a Zeiss AxioObserver Z 1 using a 380 nm filter with 200 × magnification. Pictures were taken in the Z-sectioning mode with an interval of 0.8–1.2 μm and subsequently analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA).

Splice form analysis of Nvtra *and* Nvdsx

Sex-specific fragments of *Nvtra* in 12-h-old embryos from untreated and RNAi-injected st^{DR} females were analysed by RT-PCR. Three samples containing ~30 embryos per treatment were collected in 100% ethanol and stored in −80°C until RNA extraction. One μl of cDNA was used in a PCR with *Nvtra* primers, NvTra_F2 and NvTra_R3, that are located in exon 2 and 3 respectively and give a fragment of 228 bp in females and three fragments of of 514 bp, 460 bp and 282 bp in males and *Nvdsx* primers NvDsxU_F3 and NvDsxM_R1 that are located in exon 4 and 6 respectively and give a product of 543 bp in males and 435 bp in females (Verhulst *et al.*, 2010).

For amplification and cDNA integrity control *Elongation factor 1α* (*EF1α*) was amplified with primer set EF1α_F1and EF1α_R1 (Verhulst *et al.*, 2010) yielding a product of 174 bp in both males and females. The PCR profile used was 35 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 45 s. PCR fragments were visualized on a 1.5% non-denaturing agarose gel and stained with ethidiumbromide.

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