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RESEARCH ARTICLE

The Role of Epigenetics in Insects in Changing Environments

Insect Molecular Biology

DNA methylation machinery is involved in development and reproduction in the viviparous pea aphid (*Acyrthosiphon pisum*)

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Abstract

Epigenetic mechanisms, such as DNA methylation, have been proposed to mediate plastic responses in insects. The pea aphid (*Acyrthosiphon pisum*), like the majority of extant aphids, displays cyclical parthenogenesis - the ability of mothers to switch the reproductive mode of their offspring from reproducing parthenogenetically to sexually in response to environmental cues. The pea aphid genome encodes two paralogs of the de novo DNA methyltransferase gene, *dnmt3a* and *dnmt3x*. Here we show, using phylogenetic analysis, that this gene duplication event occurred at least 150 million years ago, likely after the divergence of the lineage leading to the Aphidomorpha (phylloxerans, adelgids and true aphids) from that leading to the scale insects (Coccomorpha) and that the two paralogs are maintained in the genomes of all aphids examined. We also show that the mRNA of both *dnmt3* paralogs is maternally expressed in the viviparous aphid ovary. During development both paralogs are expressed in the germ cells of embryos beginning at stage 5 and persisting throughout development. Treatment with 5-azactyidine, a chemical that generally inhibits the DNA methylation machinery, leads to defects of oocytes and early-stage embryos and causes a proportion of later stage embryos to be born dead or die soon after birth. These phenotypes suggest a role for DNA methyltransferases in reproduction, consistent with that seen in other insects. Taking the vast evolutionary history of the *dnmt3* paralogs, and the localisation of their mRNAs in the ovary, we suggest there is a role for *dnmt3a* and/or *dnmt3x* in early development, and a role for DNA methylation machinery in reproduction and development of the viviparous pea aphid.

KEYWORDS
aphid, development, DNA methyltransferase, gene duplication, reproductive plasticity

INTRODUCTION

Aphids are hemimetabolous insects that are responsible for huge economic loss through virus-mediated and direct damage to crop plants (Brault et al., 2010; Guerrieri & Digilio, 2008). This is due, at least in part, to their ability to reproduce via viviparous parthenogenesis, which allows them to quickly amass large populations of genetically identical, live-birthed daughters (Dixon, 1985). While most extant aphids reproduce parthenogenetically in the spring and summer, they parthenogenetically birth oviparous sexual females and males in the autumn and winter, triggered as days become shorter and colder (Moran, 1992; Simon et al., 2002). This life-history strategy is known as cyclical parthenogenesis, and is a transgenerational polyphenism (Ogawa & Miura, 2014). These sexual aphids mate and produce overwintering eggs which hatch post diapause as viviparous parthenogenetically reproducing daughters in the spring, and the cycle continues

(Dixon, 2012; Ogawa & Miura, 2014). Sexual reproduction has been totally lost in approximately 3% of aphid species (Dixon, 2012) and 30–40% of populations of aphid species show variation in reproductive mode which includes obligate parthenogenesis (Defendini et al., 2023; Dixon, 2012; Jaquiery et al., 2014; Moran, 1992). Cyclical parthenogenesis is the ancestral reproductive mode of the Aphidomorpha (phylloxerans, adelgids and true aphids), which evolved approximately 220–210 million years ago (mya) (fossil record (Grimaldi & Engel, 2005), 298 ± 37 mya based on phylogenomic analyses (Johnson et al., 2018)) from an ancestor that was oviparous (Gavrilov-Zimin, 2021) and sexually reproducing (Dixon, 2012). True aphids (Aphidoidea) subsequently evolved viviparity (Davis, 2012) (development occurring inside the mother) between 170 and 150 mya (fossil record Grimaldi & Engel, 2005, 106 ± 29 mya based on phylogenomic analyses Johnson et al., 2018). Cyclical parthenogenesis and vivparity are thus both evolutionary innovations in the aphid lineage. But the mechanisms that control the plasticity of reproductive mode and ovary development in the offspring are largely unknown. Understanding the genes and molecular processes that underpin these innovations is key to our understanding of how this novelty evolves and may also yield routes to develop novel pest control strategies.

Epigenetic mechanisms, heritable and environmentally responsive changes to a genome, that are not direct changes to DNA sequence, have been proposed as possible elements mediating plastic responses (Duncan et al., 2022; Richard et al., 2019; Richard et al., 2021). DNA methylation is one such mechanism which involves the reversible addition of methyl groups to nucleotides, primarily to cytosines in CpG dinucleotides (Bewick et al., 2017; Robertson, 2005). The functions of DNA methylation are well understood in mammals where CpG methylation is typically constrained to regulatory regions of genes and downregulates gene expression (Li & Zhang, 2014). Conversely, relatively little is known about the role of DNA methylation in insects, particularly hemimetabolous insects (Bewick et al., 2017; Dixon & Matz, 2022; Duncan et al., 2022; Provataris et al., 2018). Insect DNA methylation occurs primarily in gene bodies and is associated more with highly conserved and stably expressed genes (Bewick et al., 2017; Lewis et al., 2020; Provataris et al., 2018; Sarda et al., 2012). Several studies have experimentally explored possible roles for DNA methylation in insect phenotypic plasticity, yet a clear general role has not been established (reviewed in Duncan et al., 2022). Intriguingly, several recent studies have strongly suggested methylation independent roles in reproduction for *dnmt1* in a range of insects (Blattodea Ventos-Alfonso et al., 2020), Hemiptera (Amukamara et al., 2020; Bewick et al., 2019; Cunningham et al., 2024; Shelby et al., 2023; Washington et al., 2021), Hymenoptera (Arsala et al., 2022; Ivasyk et al., 2023; Zwier et al., 2012), Lepidoptera (Li, Hu, et al., 2019), Coleoptera (Gegner et al., 2020; Schulz et al., 2018), but it is unclear at this time whether similar non-methylation functions might exist for *Dnmt3*.

The pea aphid (*Acyrthosiphon pisum*) has a complete suite of genes encoding the DNA methylation machinery, including one copy of *DNA methyltransferase 1* (*dnmt1*), two paralogs of *DNA methyltransferase 3* (*dnmt3*): *dnmt3a* and *dnmt3x* (Walsh et al., 2010) and a single copy of the putative demethylation enzyme *tet methylcytosine dioxygenase 1* (*tet1*) (Duncan et al., 2022). In the case of the pea aphid *dnmt3* paralogs, *dnmt3a* has been suggested to be a functional DNA methyltransferase, while *dnmt3x* appears to have diversified, and is lacking some of the key domains thought to be necessary for carrying out DNA methylation (Walsh et al., 2010). This raises the possibility that the duplication of *dnmt3* that gave rise to *dnmt3a* and *dnmt3x* allowed for sub-functionalisation or neo-functionalisation (of *dnmt3x*) and a possible role in the novel reproductive and developmental strategy of cyclical viviparous parthenogenetic reproduction.

Here, we demonstrate that the *dnmt3* gene duplication occurred after the split between Aphidomorpha (phylloxerans, adelgids and true aphids) and Coccomorpha (scale insects) 220–210 mya but prior to the split of phylloxerans-adelgids and true aphids 170–150 mya (Grimaldi & Engel, 2005). We show that both *dnmt3* paralogs are expressed in the ovaries and developing embryos of the viviparous asexual pea aphid. Using an inhibitor of the DNA methylation machinery, 5-azacytidine, we demonstrate that DNA methyltransferases have key roles in oogenesis and embryogenesis in the pea aphid.

METHODS

Aphid husbandry

Pea aphids (*Acyrthosiphon pisum*), clone N116 (Kanvil et al., 2014) were maintained on broad bean (*Vicia faba*) plants in a Panasonic growth chamber (352H-PE) under long-day conditions, 16L:8D at 20°C.

Phylogenetic analysis of *dnmt3a* and *dnmt3x*

Pea aphid *dnmt3a* (NCBI, XP_016662566.1) and *dnmt3x* (NCBI, XP_029348651.1) sequences) were used to search for homologues in the genomes and transcriptomes of 48 insect species (Table S1), using BLASTp and tBLASTn (using an E-value cut-off of $E < 1 \times 10^{-20}$) (Altschul et al., 1990; Camacho et al., 2009). Target databases were NCBI's protein and nucleotide collection as well as the transcriptome shotgun assembly databases, AphidBase (https://bipaa.genouest.org/is/aphidbase/) and i5k (https://i5k.nal.usda.gov/webapp/blast/ (Poelchau et al., 2015).

Protein sequences were aligned using Clustal Omega (Sievers et al., 2011). Gblocks (Talavera & Castresana, 2007) was then used to remove poorly aligned regions, using a maximum number of contiguous non-conserved positions of 20, and otherwise default parameters. The Gblocks output was then realigned using Clustal Omega (Sievers et al., 2011). MrBayes (Huelsenbeck & Ronquist, 2001) was then used to carry out Bayesian inference by Markov chain Monte Carlo simulation, using a Poisson amino acid model, a burninfrac value of 0.25, a samplefreq value of 500 and 4 million iterations, mixed models and default priors. Consensus trees were visualised using Figtree (Rambaut, 2010).

Dissection and fixation

Adult asexual pea aphids were collected from plants the day of dissection, and ovaries were dissected under a dissecting microscope in cold Phosphate Buffered Saline (PBS) using fine forceps. Tissue was collected into microcentrifuge tubes containing PBS on ice and fixed for 1 h in a mix of 50:40:10 PBS:heptane:formaldehyde (37%) on a nutator. The solution was removed and replaced with ice cold methanol, following two washes. Tissue in methanol was subsequently stored at −20°C.

Hybridisation chain reaction (HCR)

Immediately preceding hybridisation chain reaction (HCR), tissue was rehydrated over a methanol series: 3:1, 1:1, then 1:3 methanol:PTw (0.3% Tween-20 in PBS) (all PTw used in subsequent steps was 0.3% Tween-20) then rinsed three times in PTw on a nutator at room temperature for 10 minutes each step. Samples were re-fixed in 4% formaldehyde in PTw for twenty minutes on a nutator and then rinsed in PTw on a nutator for 10 minutes three times. To permeabilise the tissue, samples were then nutated for 45 minutes in detergent solution (1% SDS, 0.5% Tween-20, 50 mM Tris–HCL (pH 7.5), 1 mM EDTA (pH 8.0), and 150 mM NaCl) at room temperature.

HCR was performed according to Molecular Instruments instructions (Choi et al., 2018), with some modifications. Permeabilised tissue, which consisted of ovaries from at least five individuals, was pre-hybridised in 150 μL HCR probe hybridisation buffer (Molecular Instruments Inc, 2.4 M Urea, 5 × sodium chloride sodium citrate (SSC), 9 mM citric acid (pH 6.0), 0.1% Tween 20, 50 μg/mL heparin, 1 X Denhardt's solution, 10% dextran sulphate) at 37°C for thirty minutes. Probe solution was prepared by adding 0.8 pmol of each sequence specific probe set to 200 μL probe hybridisation buffer (Molecular Instruments Inc.) at 37°C. Pre-hybridisation solution was replaced with the prepared probe solution and incubated overnight (16–18 h) at 37°C. Excess probe was removed by washing four times, each for fifteen minutes in 100 μL preheated probe wash buffer (Molecular Instruments Inc, (2.4 M Urea, 5X SSC, 9 mM citric acid (pH 6.0), 0.1% Tween, 50 μg/mL heparin)) at 37°C. Tissue was then washed three times for five minutes in 500 μL 5 × SSCT (0.1% Tween-20 in 5 × SSC) at room temperature on a nutator. Samples were incubated with 100 μL amplification buffer (Molecular Instruments Inc, 5X SSC, 0.1% Tween 20, 10% dextran sulphate) for thirty minutes at room temperature. Simultaneously, 6 pmol of hairpin h1 and 6 pmol of hairpin h2 (2 μL of a 3-μM stock, Molecular Instruments Inc) were heated to 95°C for 90 seconds in a thermal cycler and allowed to cool over thirty minutes protected from light at room temperature. Hairpins (B2-546 for *Ap-dnmt3a*, B3-546 for *Ap-dnmt3x*, B3-546 for *Ap-wg and* B4-647 for *Ap-vasa*; Molecular Instruments Inc) were added to samples, and they were incubated overnight in the dark at room temperature.

The hairpin solution was removed, and excess hairpin was removed by nutating with 500 μL 5 × SSCT at room temperature in the dark, twice for five minutes. Samples were washed twice more with 5 × SSCT as above for thirty minutes each wash, with the first wash including 5 μg/mL DAPI (4′,6-diamidino-2-phenylindole; ThermoFisher Scientific) to stain nuclei. The two 30-min washes were followed by a final five-minute wash. All solution was then replaced with 1 mL 70% ultrapure glycerol (ThermoFisher Scientific) and samples were stored protected from the light at 4°C for at least 12 hours prior to imaging. Tissue was mounted on glass slides in a small amount of 70% glycerol with a drop of SlowFade Diamond Antifade Mountant (ThermoFisher Scientific) and stored in the dark until imaging by confocal microscopy. Images were acquired within a week of carrying out HCR, using a Zeiss LSM 880 upright confocal microscope (Zeiss) and the Zen Black software (Zeiss). Images were processed using Zen Blue (v3.1, Zeiss). All HCRs were repeated at least three times independently to ensure the robustness of the expression patterns reported.

Chemical inhibition of DNA methyltransferases

Asexual pea aphids were reared on an established artificial diet (Kunkel, 1977; Prosser & Douglas, 1992) (Table S2). Artificial diet was stored in single use aliquots at −20°C.

For treatment, preparations of artificial diet were thawed and supplemented with 5-azacytidine (supplied in powder form and dissolved in DMSO) to a final concentration of 50 μM (Acros Organics, 226,620,500) or an equivalent amount of DMSO for the control diet. Notably, 50 μM 5-azacytidine did not cause significantly increased mortality compared with DMSO or water (Figure S1); 10 μL of artificial diet was transferred into each of the eight wells of a sterile PCR tube cap strip. These strips were then pushed gently liquid side down into strips of parafilm that had been sterilised with 70% ethanol, allowed to dry, then stretched over a 70% ethanol sterilised 96-well PCR tube holder to enable the aphids to feed. Fourth instar (L4 nymph) parthenogenetic pea aphids were collected immediately prior to initiation of feeding. L4 nymphs were identified based primarily on cauda length relative to width at its base (Diamond & Levitis, 2016). Fourth instar nymphs were selected to minimise variation due to developmental stage (Diamond & Levitis, 2016) and to allow the exclusion of any morphs that were destined to be winged, as winged morphs have a lower reproductive output (Brisson, 2010). L4 aphids were transferred individually to the wells of a transparent 96-well microplate. The parafilm-strip cap complexes were then carefully removed and transferred to the 96-well microplates containing aphids, enclosing the aphids. Aphids were maintained in LD conditions (16L:8D, 20°C, 70% RH). The artificial diet solution was replaced daily by preparing fresh parafilm-strip cap complexes. Before replacing the solution, aphids were dislodged from the parafilm by gently stroking with a paintbrush, so they were able to withdraw their stylet. After six days of feeding on supplemented artificial diets, aphids were dissected and processed (as described above), and ovaries were used in HCR (as described above), using *Ap-vasa* (NCBI XM_001948573.5) and *Ap-wingless* (*Ap-wg*; NCBI XM_001945260.5) probes (Molecular Instruments Inc). Additionally, a second group of six-day fed aphid

ovaries were stained with Alexa Fluor phalloidin-488 (Cell Signalling Technologies) and DAPI (ThermoFisher Scientific). Phalloidin staining was carried out by first fixing ovaries in 50:40:10 PBS:heptane:formaldehyde (37%) for forty-five minutes, then washing for five minutes in PTw (0.3% Tween-20) five times and incubating with PTw (0.3% Tween-20) for one hour. After incubation, ovaries were washed for five minutes with PTw (0.3% Tween-20) and a solution containing 10 μL Alexa fluor 488 phalloidin (6.6-μM stock, Cell Signalling Technologies) in 200 μL total volume PTw (0.3% Tween-20) was applied. Ovaries were then incubated in the dark for forty-five minutes, after which 800 μL PTw (0.3% Tween-20) and 1 μL DAPI were added to the ovaries in solution, and ovaries were then incubated for a further thirty minutes in the dark. Following, ovaries were washed as before three times, and then once for five minutes in PBS. Ovaries were stored in 70% glycerol before mounting in a small amount of 70% glycerol with a drop of SlowFade™ Diamond Antifade Mountant (ThermoFisher Scientific) and imaged within two days using a Zeiss LSM 880 upright confocal microscope.

To understand the effects of this chemical treatment on the life history of this population, a group of aphids were fed azacytidine-supplemented or DMSO-control diets for six days. Aphids were then moved to leaf-agar plates (one *Vicia faba* leaf cut close to the petiole using a sterile razor blade embedded in 5 mL of 2% agar supplemented with 1 g/L Miracle-Gro (Miura et al., 2003) and 0.03% Methyl 4-hydroxybenzoate (to inhibit fungal growth) in a 55 × 15 mm petri dish) and monitored for another 14 days (20 days total) and their survival, offspring production, offspring phenotype and offspring survivability were assessed daily. No more than ten aphids (the focal aphid plus nine offspring) were kept on any one leaf-agar plate at a time, with progeny being spread across several plates where necessary.

Data analysis

Statistical analyses were performed, and graphs were produced using R version 4.1.2. A Cox proportional hazard model was run to assess differences in lifespan between 5-azacytidine and DMSO-control focal aphids using the *survival* package. Differences between 5-azacytidine and DMSO-control aphids in their daily reproductive output were assessed by fitting zero-inflated Poisson GLMMs fitted with a log link function, aphid ID as a random effect and treatment and day as the fixed effects, and their interaction. The main effects were assessed separately from the interaction and then included with the interaction. This was followed by performing likelihood ratio tests (LRT) comparing the full models with null models with the fixed effects (or fixed effects and their interaction) dropped (using the lmtest package); the emmeans package was then used to perform pair-wise contrasts (Tukey) for comparisons of treatments for specific days. For day sixteen, a separate analysis was performed, owing to high SE associated with all values for 5-azacytidine being 0 on this day; a full model including all interactions between treatment and day was compared by LRT with an equal model with the interaction effect of treatment on day sixteen removed. The data were truncated to remove entries prior to initiation of reproduction by a given aphid (all only being able to be 0). Both time from L4 to reproduction and production of disturbed (stillborn) nymphs by 5-azacytidine and DMSO-control aphids were assessed by Wilcoxon rank sum test with continuity correction. General survival of nymphs (produced between days six and twenty) were compared by Wilcoxon rank sum test. And fecundity of the offspring of focal aphids (collectively, as pooled groups of offspring) was compared between groups by running a linear model, with aphid ID and day as random effects and treatment as a fixed effect, then performing an LRT as above.

RESULTS

Dnmt3a and *dnmt3x* arose from an ancient Aphidomorpha specific duplication

Aphid genomes generally show a high number of gene duplications (International Aphid Genomics, 2010; Li, Park, et al., 2019; Mathers et al., 2017), but many of these are lineage-specific or species-specific duplications (International Aphid Genomics, 2010; Mathers et al., 2017). To investigate the evolutionary history of the duplication event giving rise to *Ap-dnmt3a* and *Ap-dnmt3x* and identify the conserved paralog of the gene, we first identified homologues of *dnmt3* in the genomes and transcriptomes of 48 insect species (Table S1).

Dnmt3 protein sequences were aligned, and a phylogeny constructed using Bayesian methods (Ronquist & Huelsenbeck, 2003, Figure 1a) robustly separates these homologues into two clades. One clade contains Ap-Dnmt3a and all other insect Dnmt3 orthologs, while the second clade contains Ap-Dnmt3x and orthologs from eight other aphid species. This separation is consistent with Ap-Dnmt3a being the more highly conserved, possibly ancestral, copy of Dnmt3, while Ap-Dnmt3x is more diverged.

The Dnmt3x clade also includes sequences from the grape phylloxera (*Daktulosphaira vitifoliae*), a representative phylloxeran species, and the gall adelgid (*Adelges cooleyi*), a representative adelgid species. Phylloxerans and adelgids diverged from the lineage leading to the true aphids approximately 170–150 mya, and the Aphidomorpha (phylloxerans, adelgids and true aphids) split from the Coccomorpha (scale insects) 220–210 mya (Grimaldi & Engel, 2005). The conservation of both paralogs in these phylloxeran and adelgid species, as well as all true aphid species examined, combined with the absence of dnmt3 genes in scale insects, indicates that the duplication giving rise to *dnmt3a* and *dnmt3x* is ancient and likely occurred between 220 and 150 mya (Grimaldi & Engel, 2005). This gene duplication likely occurred after the divergence of the lineages giving rise to the Coccomorpha, where *dnmt3* has been lost (Figure 1b), and the Aphidomorpha (phylloxerans, adelgids and true aphids), all of which have at least two copies of *dnmt3*: *dnmt3a* and *dnmt3x*. However, it is also possible that the duplication occurred prior to this in the lineage leading to the Coccomorpha/Aphidomorpha after the divergence of the lineage leading to the Aleyrodidae (whiteflies), and both duplicates

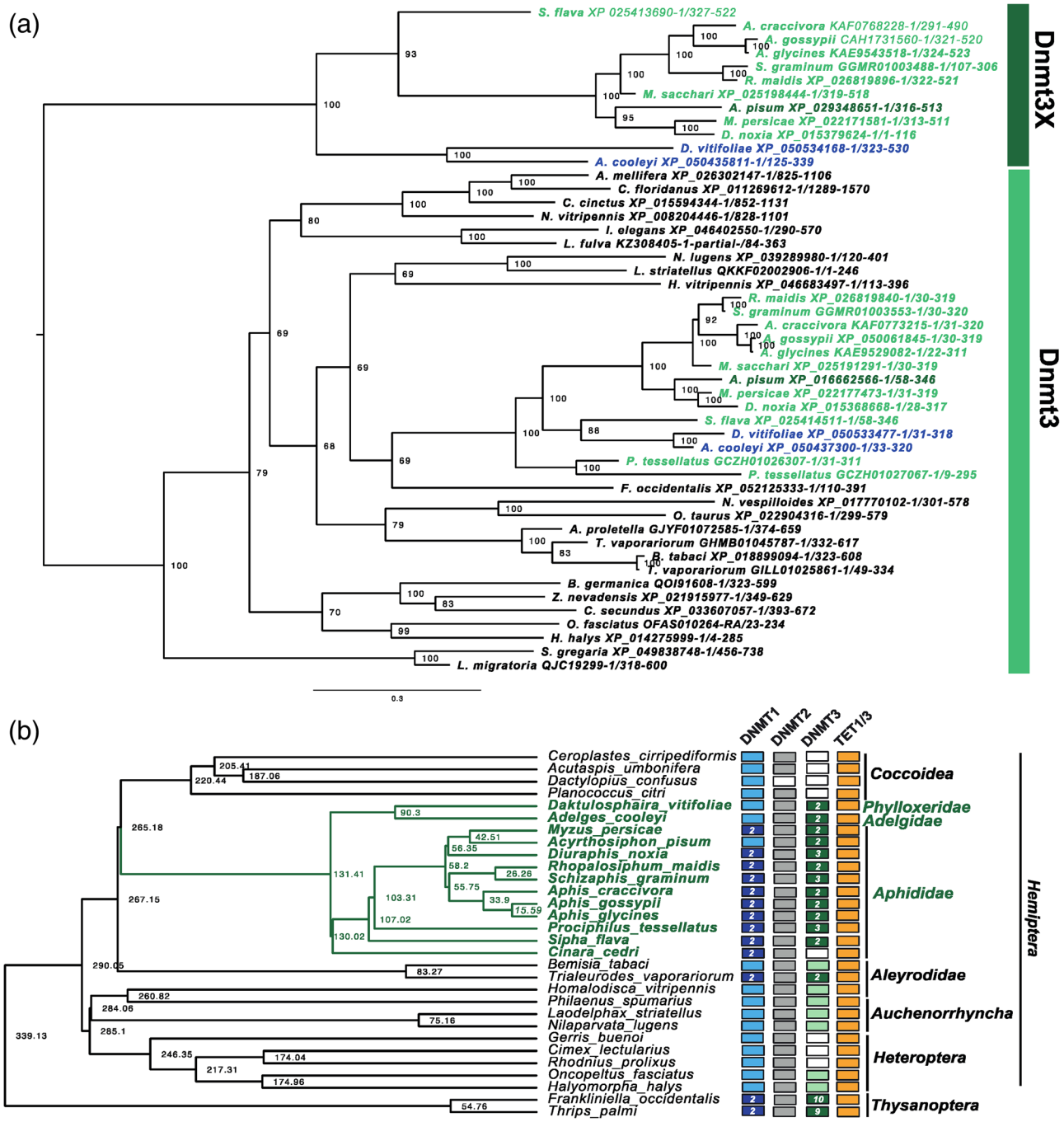

FIGURE 1 Evolutionary History of the Dnmt3 paralogs. (a) Bayesian phylogeny of insect Dnmt3 proteins from 48 insect species. The phylogenetic tree is midpoint rooted and posterior probabilities are shown at nodes. This analysis reveals two well supported clades, an ancestral clade corresponding to the conserved Dnmt3 protein (light green box) containing orthologs from a wide phylogenetic range of insect species, and the second containing the dnmt3 paralogs (dark green box). Aphid species are shown in green text while Adelogidea and Phylloxeroidea are shown in blue text. Full species names and protein accessions are provided in Supplementary File 1. (b) Conservation of genes involved in DNA methylation across Hemiptera and Thysanoptera. The phylogenetic relationships between species and divergence times (shown at nodes) are based on TimeTree of Life (Kumar et al., 2022). Lighter colours indicate the presence of a single homologue in the genome, while darker colours indicate the presence of paralogs (the number of paralogs is indicated). *dnmt1*, *dnmt2* and *tet1* are generally very well conserved across the Hemiptera and Thysanoptera although duplicates of *dnmt1* are often seen, which may indicate the potential for neo or sub-functionalisation of these paralogs. In contrast, *dnmt2* and *tet1* are never duplicated. *dnmt3* exhibits more variability (full figure showing a wider range of insect species is shown in Figure S2).

were subsequently lost in the lineage leading to the Coccomorpha. Either way these paralogs have been stably maintained in the genomes of these species for at least 150 million years (Grimaldi & Engel, 2005). Although the two *dnmt3* paralogs are present on the same chromosome in pea aphid (X) they are separated by 151 protein coding genes and 6.19 Mbp, and there was no detectable synteny

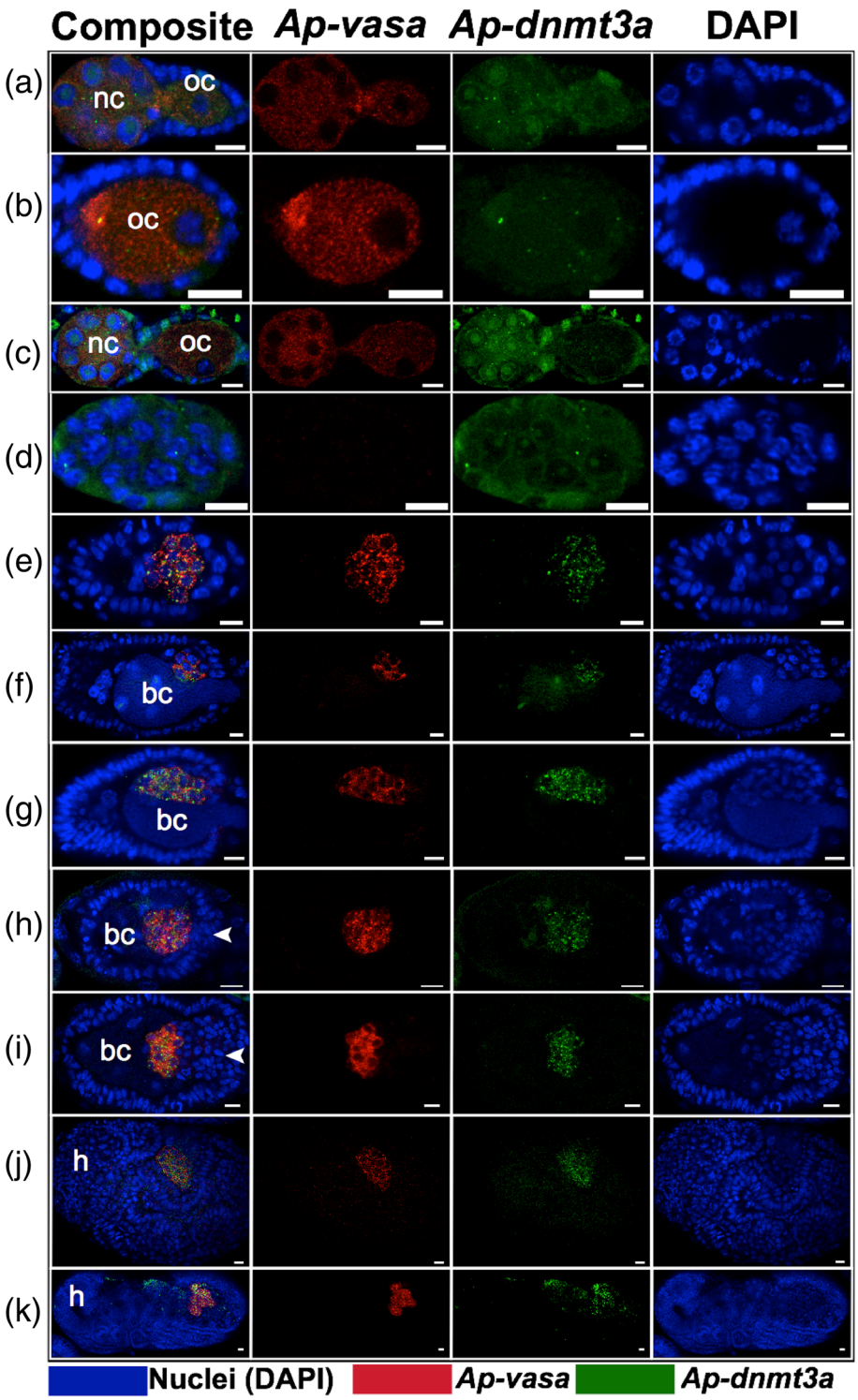

FIGURE 2 Expression of *Ap-vasa and Ap-dnmt3a* during viviparous oogenesis (a–c) and embryogenesis (d–k). a–c oogenesis: *Ap-vasa* and *Ap-dnmt3a* are maternally provided and mRNAs are detected in the germarium (a) and at stage 0 (b) through to stage 1 of oogenesis (b). *Ap-vasa* mRNA is strongly localised to the anterior of the developing oocyte (as has been previously reported (Chang et al., 2007); however, this is not observed for *Ap-dnmt3*. d–k embryogenesis: Between late oogenesis and early embryogenesis (Stage 3, d), maternal mRNAs are cleared from the embryo, and no signal for either *Ap-vasa* nor *Ap-dnmt3a* can be detected. Embryonic expression of both *Ap-vasa* and *Ap-dnmt3a* is detected early in stage 6 after the cellular blastoderm has formed in the presumptive germ cells (e). The co-localisation of *Ap-dnmt3* with *Ap-vasa* persists throughout the rest of development, including as the germ cells migrate dorsally to accommodate the invading endosymbiotic bacteria at stage 7 (f,g). The co-expression of *Ap-dnmt3* with *Ap-vasa* continues through the beginning of gastrulation (Stage 8, h), as the bacteria have finished invading and the germ band begins to invaginate (arrowhead). This co-expression is also seen at Stage 9 (germ band invagination, arrowhead) (i), stage 12 (j) and post-katatrepsis as the germ band retracts at stage 17 (k). Arrowhead = invaginating germ band, bc = bacteriocytes, h = head, nc = nurse cells, oc = oocyte. Scale bars represent 10 μm.

even between closely related species (Figure S3), consistent with this duplication being ancient.

The dnmt3a paralog identified in Aphidomorpha is clustered with the other insect dnmt3 orthologs (Figure 1) suggesting it may be more similar to the ancestral gene copy (Figure 1). Analysis of domain architecture (Figure S4) and intron exon structure (Figure S5) shows that proteins in the Dnmt3x paralog group retain more organisational similarity to the Orthopteran Dnmt3 protein sequences, which may suggest sub-functionalisation of the ancestral gene between these two paralogs (Figures S4 and S5).

Ap-dnmt3a, *ap-dnmt3x* and *ap-vasa* mRNAs co-localise in the germ cells of developing embryos and the germaria

To examine possible sub-functionalisation and/or neo-functionalisation of Dnmt3 paralogs, we investigated the expression of *Ap-dnmt3a* and *Ap-dnmt3x* during development with mRNA localisation using in situ hybridisation chain reaction (HCR). Expression of both *dnmt3* paralogs consistently co-localises with *Ap-vasa* (a conserved marker of germ cells Chang et al., 2006, 2007) indicating that these *dnmt3* paralogs are expressed in the developing germ cells of the pea aphid (Figures 2 and 3).

Ap-dnmt3a (Figure 2a–c) and *Ap-dnmt3x* (Figure 3a,b) mRNAs are both maternally provided and are weakly detected in the germaria and in early oocytes (stages 0, 1). *Ap-vasa* localises to the anterior of the developing oocyte at stage 1 (oocytes have just been pinched off from the germarium by the follicle cells but are still connected to the germarium by the trophic core) (Figure 3b) and we see similar, albeit weak, localisation for *Ap-dnmt3x* (Figure 3b), but not for *Ap-dnmt3A* (Figure 2b). *Ap-vasa*, *Ap-dnmt3a* and *Ap-dnmt3x* mRNAs are not detected at stages 2–3 of development (Figure 2d; Figure 3c), likely as a result of clearance of maternal mRNAs. *Ap-vasa* mRNA is not detected again until stage 5, where it is located in the cytoplasm surrounding nuclei once they have localised to the periphery, with increased signal intensity at the posterior end where the presumptive germ cells are located (Figure 3d). At the same stage, and before the germ cells are specified at the posterior end of the embryo, weak punctate expression of *Ap-dnmt3x* mRNA is detected around the nuclei around the embryo's periphery, across the embryo but with greater signal intensity at the posterior end where the presumptive germ cells are located (Figure 3d). At stage 6, the germ cells are specified and split the embryo into a central syncytium and posterior syncytium. From this stage onwards, *Ap-vasa* mRNA expression is restricted to the germ cells, and *Ap-dnmt3a* and *Ap-dnmt3x* mRNAs both co-localise with *Ap-vasa* as the germ cells migrate as the result of infiltration of the endosymbiotic bacteria at stage 7 (Figure 2f,g; Figure 3g) and as the germ band invaginates beginning at stage 8/9 (Figure 2h,i and Figure 3h). This co-expression persists throughout mid-late development (Figure 2j,k; Figure 3i). The tight temporal and spatial co-localisation of *Ap-dnmt3a*, *Ap-dnmt3x* with *Ap-vasa*, which has a known and conserved role in germ-cell specification, may indicate that the *Ap-dnmt3* genes may also have a role in this process.

Treatment with the DNA methylation inhibitor 5-azacytidine treatment causes defects in reproduction and development in viviparous pea aphids

To examine potential roles of DNA methylation in pea aphid reproduction and development, we treated aphids with the non-specific DNA methyltransferase inhibitor 5-azacytidine (Stresemann & Lyko, 2008; Yang et al., 2023). We found that while having equal lifespans (Cox proportional hazards model, $p = 0.7$, Figure S1), and exhibiting no difference in the first day of reproduction (5-azacytidine mean = 3.71 days, DMSO mean = 4.2 days, Wilcoxon rank sum test: $W = 25.5$, $p = 0.3531$, Figure 4a), 5-azacytidine treated aphids produced significantly fewer nymphs per day than DMSO-control aphids during the twenty day period, specifically on days thirteen, fourteen, fifteen and sixteen, where there were significant effects of the interaction between treatment and day (main effects, GLMM, LRT: $\chi^2 = 55.261$, $p = 2.12e\text{-}05$, df = 21, 2; treatment: estimate = 0.43687, $p = 7.01e\text{-}05$, se = 0.10988; interaction, GLMM, LRT: $\chi^2 = 116.63$, $p = 1.939e\text{-}10$, df = 38, 2; treatment-day interaction effects: day 13, estimate = −1.4455, $p = 0.0013$, se = 4.50e-01, day 14, estimate = −2.8429, $p = 0.0053$, se = 1.02e+00, day 15, estimate = −1.3832, $p = 0.0120$, se = 5.50e-01; treatment-day 16 interaction GLMM, LRT: $\chi^2 = 15.824$, $p = 6.95e\text{-}05$, df = 38, 37; estimates on log scale; Figure 4b).

Treatment with 5-azacytidine resulted in the production of 'stillborn' nymphs, a phenotype that was never seen in the DMSO controls. Stillborn nymphs were either dead at birth or died shortly after birth. The gross morphology of stillborn nymphs was normal, and the only obvious defect seen was an inability to extend their legs, perhaps owing to a failure of the serosa to rupture (Figure 5a). Notably, 75% of all azacytidine treated aphids produced at least one stillborn nymph (Figure 5a), and no stillborn nymphs were observed in the DMSO control (azacytidine treated aphids produced significantly more stillborn nymphs than did DMSO-control aphids, Wilcoxon rank sum test, $W = 3995$, $p = 0.001441$; Figure 5a). Outside of the production of stillborn nymphs, all nymphs produced by 5-azacytidine treated adults, and DMSO-control adults appeared morphologically normal, and there was no difference in the mortality of the offspring of azacytidine-treated and control aphid nymphs (proportion of total nymphs dead, Wilcoxon, $W = 49$, $p = 0.1874$), or the reproductive output of these offspring (LM, LRT: $\chi^2 = 0.8084$, $p = 0.3686$, df = 3, 2; Figure S6). Stillborn nymphs were also not observed among the offspring of the offspring of treated aphids.

Each aphid that produced stillborn nymphs had between one and four total stillborn nymphs between days twelve and fourteen, but mostly appearing on day twelve (six days after the cessation of treatment) (Figure 5b). Temporally, this coincided with the period of

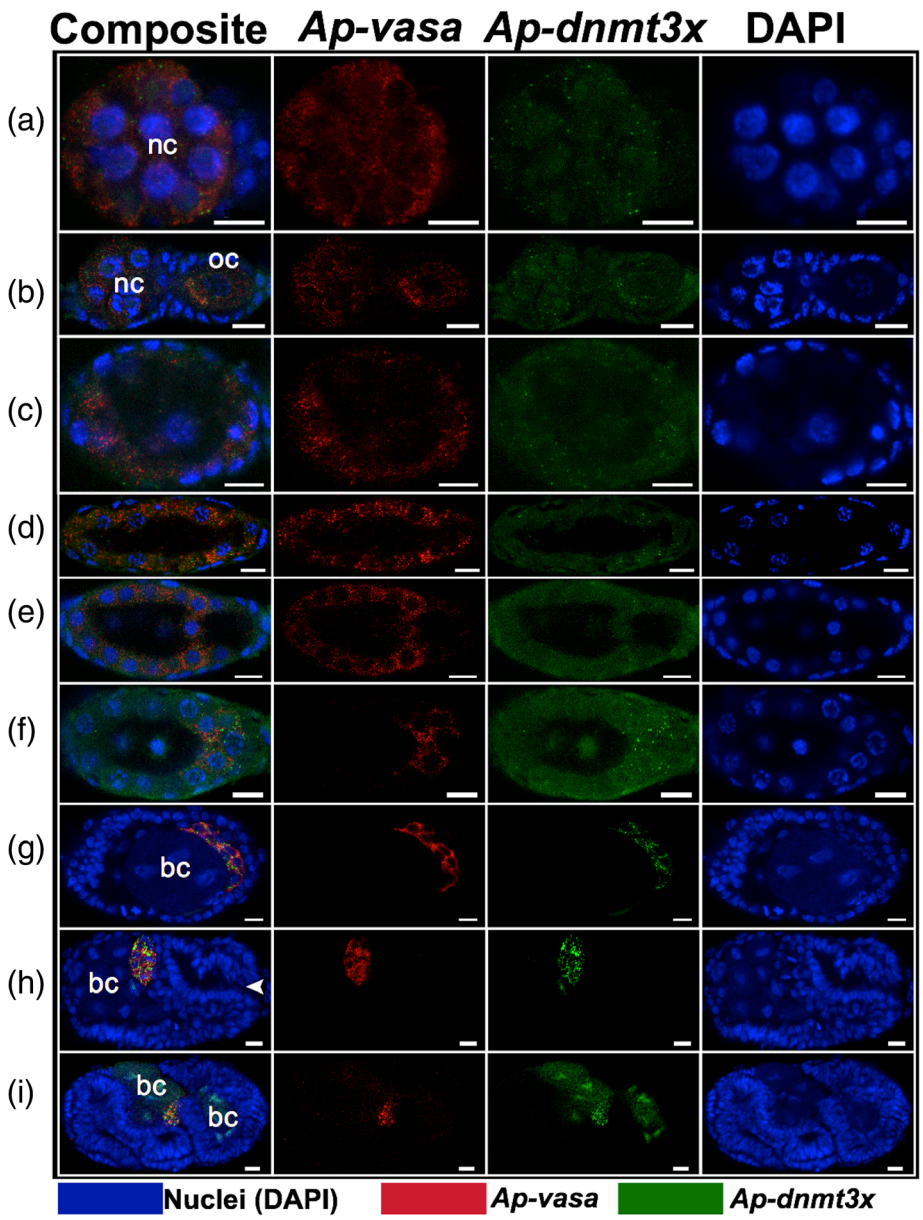

FIGURE 3 Expression of *Ap-vasa and Ap-dnmt3x* during viviparous oogenesis (a,b) and embryogenesis (c–j). a,b oogenesis: *Ap-vasa* and *Ap-dnmt3x* are maternally provided and mRNAs for *Ap-dnmt3x* are weakly detected in the germarium (a) and at stage 1 of oogenesis (b), where *Ap-vasa* mRNA and *Ap-dnmt3x* mRNAs are localised to the anterior of the developing oocyte (as has been previously reported for *Ap-vasa* (Chang et al., 2007). c–i embryogenesis: Between late oogenesis and early embryogenesis (Stage 2, c), maternal mRNAs are cleared from the embryo and no signal for either *Ap-vasa* nor *Ap-dnmt3x* can be detected. Embryonic expression of *Ap-vasa* is detected ubiquitously throughout the cellular blastoderm (stage 5, d), while punctate expression of *Ap-dnmt3x* is detected in cells of the posterior blastoderm only (d). As the germ cells are specified (stage 6 (e,f)), the expression of both *Ap-vasa* and *Ap-dnmt3x* intensifies and is localised to the presumptive germ cells. The co-localisation of *Ap-dnmt3x* with *Ap-vasa* persists throughout the rest of development, including as the germ cells migrate dorsally to accommodate the invading endosymbiotic bacteria at stage 7 (g), during gastrulation (Stage 8, h), as bacteria have finished invading and the germ band invaginates (arrowhead) and through stage 11 and 12 (i). Arrowhead = invaginating germ band, bc = bacteriocytes, nc = nurse cells, oc = oocyte. Scale bars represent 10 μm.

reduced reproduction seen in 5-azacytidine treated aphids (Figure 4b). It is interesting to note that, while nymph production slowed down during the production of stillborn nymphs, it generally did not stop completely. And, where these stillborn nymphs appeared more than once during this period, they did so in chains uninterrupted by normal nymphs (except for one stillborn nymph produced by a single aphid on day 7), suggesting that 5-azacytidine treatment was affecting a critical period in development essential for embryo maturation 6–8 days prior to birth, or that 5-azacytidine has a high level of stability in the pea aphid.

The decreased reproductive rate in 5-azacytidine treated aphids (Figure 4b) may indicate defects in early development that result in

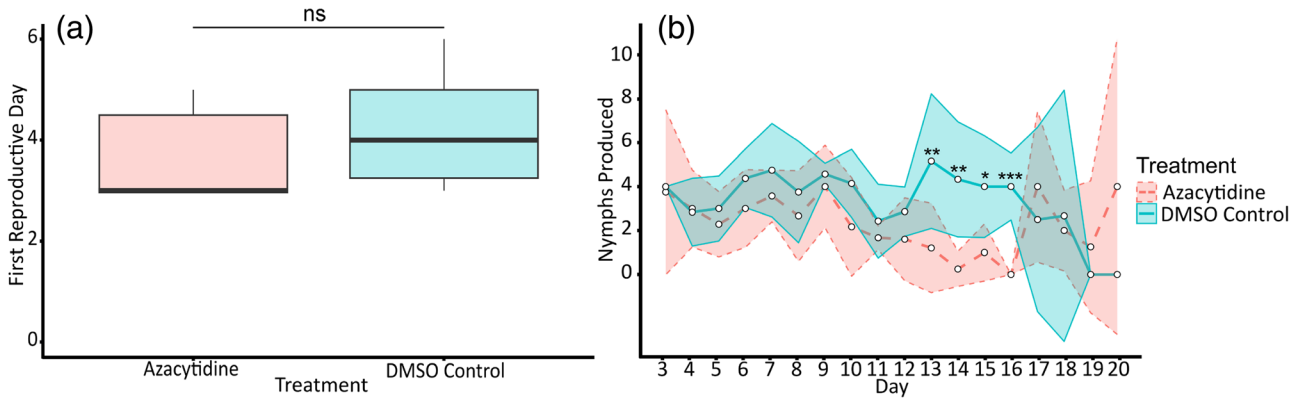

FIGURE 4 5-azacytidine treatment alters reproductive rate in viviparous pea aphids. (a) Boxplots showing the first day reproduction was observed for 5-azacytidine and DMSO-treated groups (ns = not significant). Reproduction initiated, at the earliest, 3 days after the onset of treatment. (b) Daily reproductive output of 5-azacytidine and DMSO-treated aphids, from day 3 (the earliest day nymphs were recorded for any aphid), until day 20 (at which point the experiment was concluded due to sample size); considering both alive and 'stillborn' nymphs. Points represent mean nymphs produced by all individuals of a treatment and ribbons indicate 95% confidence intervals, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Sixteen 5-azacytidine and fifteen DMSO treated aphids were studied (at day 0), though these numbers decreased during the study period.

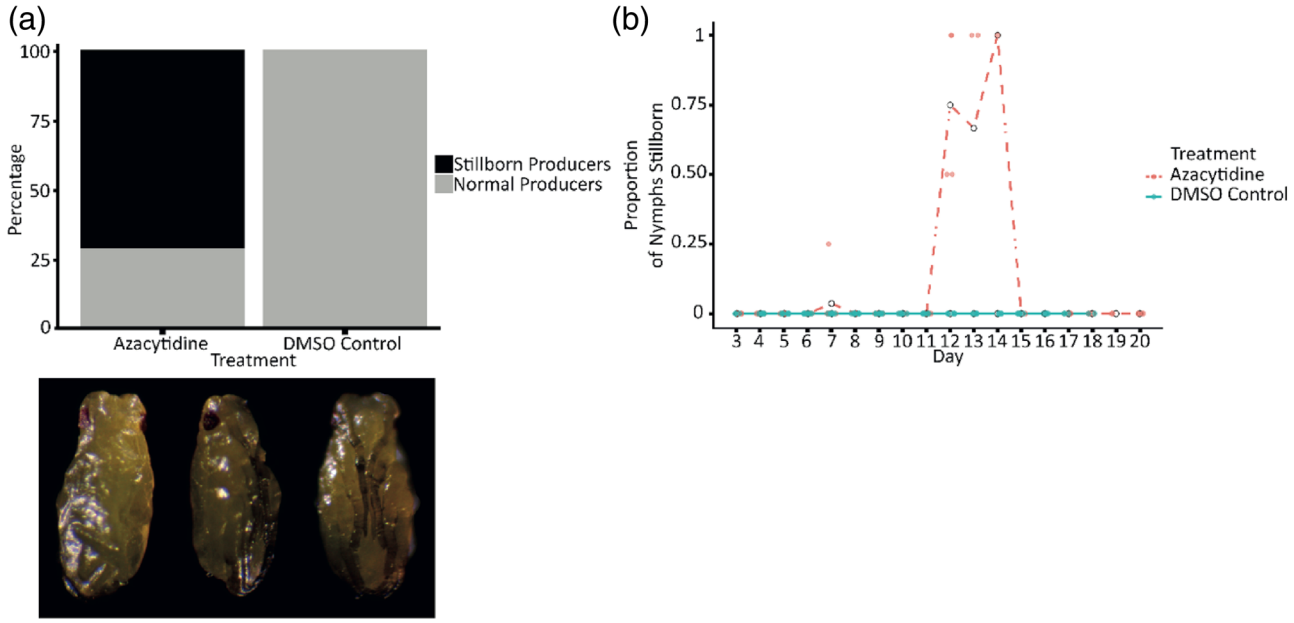

FIGURE 5 5-azacytidine treatment causes mature embryos to be stillborn in viviparous pea aphids. (a) Percentage of all 5-azacytidine (50 μM) treated and control (DMSO) aphids that produced 'stillborn' (bottom panel: stillborn nymphs were either dead at birth or shortly after birth and their gross morphology was normal) aphids after treatment from day 0 to day 6. Stillborn offspring were only produced by 5-azacytidine treated aphids. (b) Daily mean proportion of stillborn nymphs for each 5-azacytidine-treated and control aphid, between day 3 (the first day nymphs were observed) and day 20. Sixteen 5-azacytidine and fifteen DMSO treated aphids were studied (at day 0), though these numbers decreased during the study period.

resorption of the oocyte or embryo (Ward & Dixon, 1982). To examine this, we dissected and stained ovaries with developing embryos with phalloidin and DAPI to identify gross morphological differences (Figure 6) and performed HCR with *Ap-vasa* and *Ap-wg* to identify specific defects in germ-cell specification and segmentation respectively (Figure 7).

After treatment for six days, the germaria, oocytes and very early embryos of azacytidine treated aphids appear unusual. In the germarium, the general morphology was disturbed with defects in the gross structure (e.g. Figure 6a, where an extrusion of cortical actin is seen originating from the germarium) as well as higher numbers of cells and disorganisation of the cells contained within the germarium

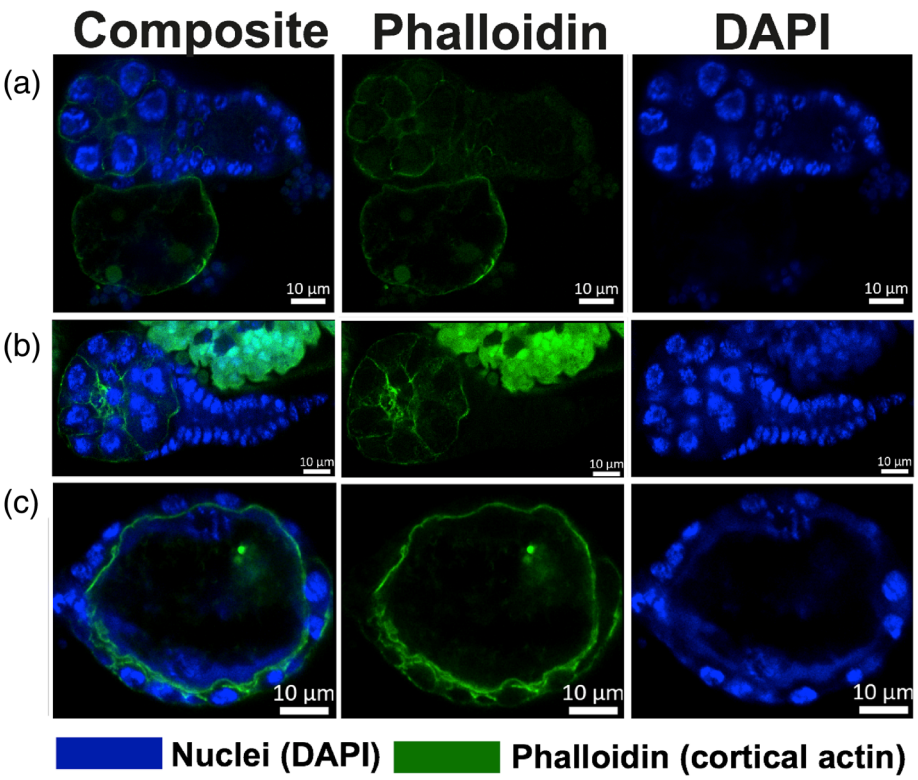

FIGURE 6 5-azacytidine treatment causes early defects in oogenesis in viviparous pea aphids. Representative images of *A. pisum* ovary samples stained with phalloidin (stains actin, green, middle) and DAPI (stains nuclei, blue, right) after aphids were treated with 5-azacytidine, an inhibitor of DNA methyltransferases. Images are single plane of focus per row, and individual channels are presented alongside composite (left). (a) Phalloidin staining reveals an unusual structure, positive for actin and appearing ectopic to the germarium and oocyte, possibly a mass of cortical actin. (b) The second row shows two adjacent oocytes and the germarium; the oocytes appear abnormal in that they do not appear to have separated from each other and form a long continuous chain. (c) The third row shows an oocyte with an abnormal size and morphology for the number of nuclei. Abnormal phenotypes were observed in multiple ovaries from 5-azacytidine-treated individuals across multiple experiments, but never observed in control individuals.

(Figure 6a,b), possibly due to an arrest in the progression of oogenesis. There is a failure of oocytes to separate from the germarium and from each other (Figure 6b) which may indicate defects in follicle cell differentiation. In other species, such as *Tribolium* (Baumer et al., 2012), differentiation of the follicle cell population is required to establish proper morphology of the egg chamber and separation of the egg chambers, although it has been suggested that aphid follicle cells do not diversify into distinct populations (Michalik et al., 2013). In severe cases, we see multiple oocytes fail to separate and form a long, continuous chain surrounded by follicle cells around their edges, this phenotype is similar to that seen with *dnmt1* RNAi in *Oncopeltus fasciatus* (Amukamara et al., 2020; Bewick et al., 2019; Washington et al., 2021).

Defects in gross morphology (Figure 6c) and gene expression (Figure 7) are observed in the early stages of embryogenesis as a result of 5-azacytidine treatment. These defects make the embryos difficult to confidently stage; however, using the expression of *Ap-vasa* as a marker of germ cell specification (Chang et al., 2007) and *Ap-wg* as a marker of posterior specification and segmentation (Duncan, Benton, & Dearden, 2013), it is clear that there is a range of phenotypes observed (Figure 7). We observe defects of cell number and organisation in the germaria (Figure 7a,b) that correspond with low levels or abnormal *Ap-vasa* expression. In some cases, we see extrusions from the early oocyte/embryo that stain positive for *Ap-vasa* (Figure 7b). We also see significant defects or delays in early embryogenesis (Figure 7c,d), with relatively normal arrangement of *Ap-vasa* and *Ap-wg* expression (Figure 7c,d), but this expression is likely occurring prematurely with less cells than we would expect staining positive for each marker (only two cells to the posterior of the embryo staining positive for *Ap-wg* adjacent to only three cells staining positive for *Ap-vasa*). Additionally, based on the expression of *Ap-vasa* and *Ap-wg* this embryo would be considered late stage 6 (blastoderm), yet the overall morphology and number of cells present in this embryo is inconsistent with a blastoderm stage embryo. More severe defects in embryogenesis as a result of 5-azacytidine treatment are also observed with some embryos displaying completely aberrant expression of *Ap-vasa* and *Ap-wg* (Figure 7e, with *Ap-wg* being expressed in a small central domain bounded by two *Ap-vasa* expression domains), consistent with defects in axis patterning.

From stage seven onwards, no defects in morphology or expression of *Ap-vasa* or *Ap-wg* were observed (data not shown). This may suggest that the activity of the DNA methylation machinery is integral

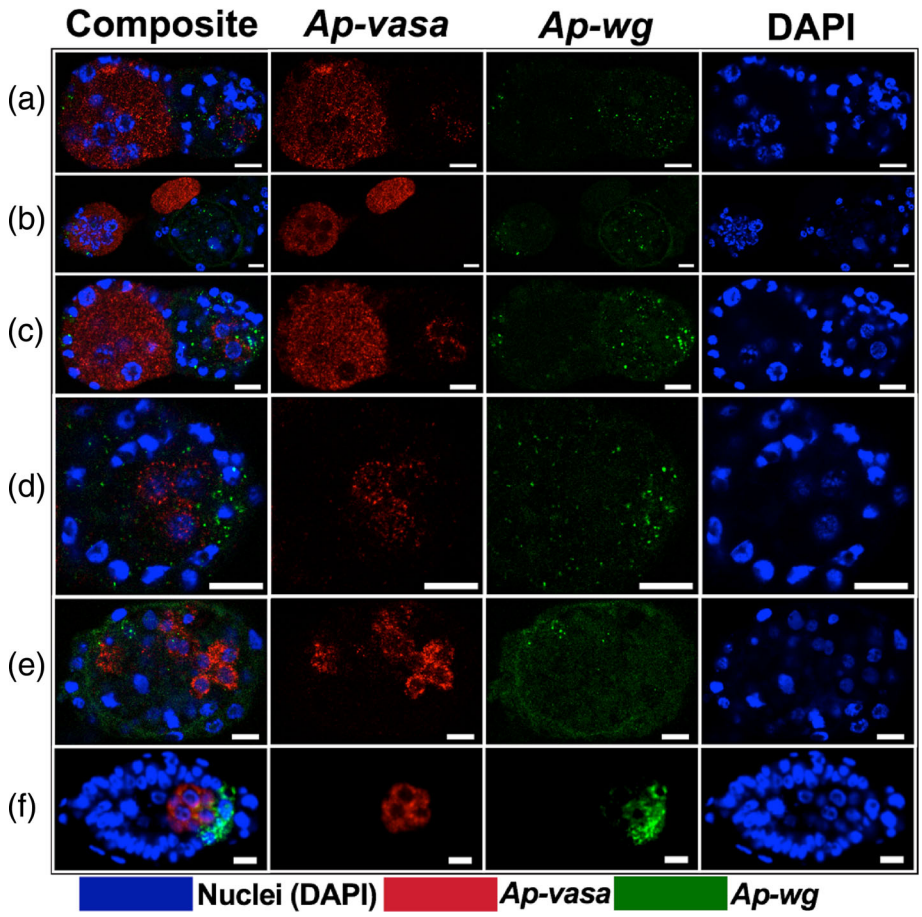

FIGURE 7 5-azacytidine treatment causes defects in early embryogenesis in viviparous pea aphids. Representative images of *A. pisum* ovary and embryo samples showing expression of *Ap-vasa* (red), *Ap-wg* (green) DAPI (stains nuclei, blue, right) after aphids were treated with 5-azacytidine, a disruptor of DNA methyltransferases. Images are single plane of focus per row, and individual channels are presented alongside composite (left). Due to the severity of defects embryos are difficult to accurately stage. (a). A malformed germarium and early oocyte (right) and a misshapen early blastoderm embryo (left). Both specimens show aberrant expression of *Ap-vasa* which is notably absent from the blastoderm stage embryo and suggests that germ cells have not been correctly specified. (b). Note the unusual arrangement of cells in the germarium (left), the embryo to the right is consistent with an early blastoderm stage embryo (stage 5) but has fewer cells. Note also the extrusion of cytoplasm from the embryo that is positive for *Ap-vasa* mRNA. (c). Shows an oocyte/ early embryo with two nuclei and a polar body visible (left), we see no localisation of *Ap-vasa* mRNA. To the right the embryo has too few cells and is round rather than elongated, yet we see expression of *Ap-vasa* and *Ap-wg* consistent with a late blastoderm stage embryo (stage 6 embryo, wild type blastoderm embryo is shown for comparison in F.). (d,e). Here we see defects in *Ap-vasa* expression that we attribute to defects in establishing the anterior–posterior and dorso-ventral axes. Rather than being restricted to the posterior, we see *Ap-vasa* expression in cells at the anterior and dorsal surface of the embryo, consistent with these cells being aberrantly specified as germ cells. There is little to no *Ap-wg* expression at the posterior, instead weak *Ap-wg* expression is observed at the anterior dorsal surface of the embryo. Scale bars represent 10 μm. Abnormal phenotypes were observed in multiple ovaries from 5-azacytidine-treated individuals across multiple experiments, but never observed in control individuals. (f). Control blastoderm stage embryo (stage 6) shows expression of *Ap-vasa* in the presumptive germ cells and *Ap-wg* in cells immediately adjacent to the presumptive germ cells at the posterior of the embryo.

only at the early stages of development, or that the effects later in development are subtle as these embryos have developed beyond a critical period.

DISCUSSION

Cyclical parthenogenesis is a novel and remarkably successful life-history strategy that is observed in most extant aphids (Brault et al., 2010; Dixon, 1985; Guerrieri & Digilio, 2008). Cyclical parthenogenesis evolved in the Aphidomorpha approximately 220–150 mya (Grimaldi & Engel, 2005) from an ancestor that was oviparous (Gavrilov-Zimin, 2021) and sexually reproducing (Dixon, 2012). This reproductive strategy allows aphid species to switch between viviparous parthenogenesis and oviparous sexual reproduction dependent on environmental cues (Ogawa & Miura, 2014). However, the mechanisms that control the plasticity of reproductive mode and ovary development in the offspring are largely unknown. Here, we investigate the evolutionary history and expression of two paralogs of the de novo methyltransferase *dnmt3* in the pea aphid. We further

characterise the function of the DNA methyltransferase proteins in oogenesis and embryogenesis using a chemical inhibitor, 5-azacytidine.

Duplicated genes have been long thought to be a source of evolutionary novelty (Ohno, 1970). Aphidomorpha, including true aphids (Fernández et al., 2020; International Aphid Genomics, 2010; Li, Park, et al., 2019; Mathers et al., 2017), phylloxerans and adelgids (Julca et al., 2020; Li et al., 2023) all have very high levels of gene duplications. Many of these gene duplications are species-specific and therefore likely to be more recent (Fernández et al., 2020), but a burst of duplication in the lineage leading to the Aphidomorpha also means that a significant proportion are ancient (occurring between 220 and 150 mya (Grimaldi & Engel, 2005)) and conserved (Julca et al., 2020; Li et al., 2023), and these duplications may have had a role in facilitating the evolution of novel and diverse life history strategies, such as parthenogenesis, within this clade.

Here, we show that the duplication that gave rise to two copies of *dnmt3* in the pea aphid (Walsh et al., 2010) likely occurred between 220 and 150 mya in the Aphidomorpha (the lineage that gave rise to the phylloxerans, adelgids and true aphids). Duplicated genes that are redundant tend to be silenced or removed from the genome (Lynch & Conery, 2000), but some duplicates are retained either because they have evolved new functions (neo-functionalisation; Ohno, 1970) or taken on part of the function of the ancestral gene (sub-functionalisation; Force et al., 1999). Orthologs of *dnmt3*, a gene that is implicated in de novo DNA methylation, are found across plants, animals and fungi (Zemach et al., 2010) with the gene evolving ~1000 million years ago (Golding, 1996). Although it is ancient, the *dnmt3* gene is also evolutionarily labile and has been lost repeatedly in multiple insect lineages, even in closely related species (Bewick et al., 2017; Duncan et al., 2022; Provataris et al., 2018). Therefore, the broad conservation of both *dnmt3* paralogs in Aphidomorpha is consistent with each having an essential function. To begin to address the possible functions of these paralogs, we examined the expression of both paralogs in viviparous pea aphid ovaries and during embryonic development (Figures 2 and 3).

We found that *Ap-dnmt3a* and *Ap-dnmt3x* mRNA co-localise temporally and spatially with *Ap-vasa* mRNA, a germline marker (Chang et al., 2006), in asexual pea aphid ovaries and throughout embryonic development. The expression of both *dnmt3* paralogs maternally and in the presumptive germ cells at the same time as *Ap-vasa* may indicate a role for these genes in germ cell specification or maturation. Dnmt3 is a positive regulator of translation, in honeybees (Kucharski et al., 2023) and mammals (Rona et al., 2016). Dnmt3 has been shown to associate with specific histone modifications through the PWWP domain (Dhayalan et al., 2010; Du et al., 2015; Kucharski et al., 2023; Rona et al., 2016), which is also present in Ap-Dnmt3x, and so it is possible that, in this context, the dnmt3 paralogs may act as regulators of germ cell specification or maintenance through transcriptional regulation.

To explore possible roles for DNA methyltransferases in the ovary, we treated asexual pea aphids with 5-azacytidine, which inhibits methyltransferase activity by causing the enzymes to irreversibly bind to DNA, after which they are degraded (Stresemann & Lyko, 2008). This treatment likely reduces the ability of DNA methyltransferases to carry out any methylation-dependent and methylation-independent functions. Although this treatment does not allow us to differentiate between the functions of Dnmt3a, Dnmt3x or Dnmt1, it can tell us the role, if any, of DNA methyltransferases in reproduction and development of the pea aphid. Exposure to 5-azacytidine led to gross morphological defects in early embryos and oocytes, and disorganisation of germaria, likely because of failures in oocyte specification, separation and production. This phenotype is broadly consistent with the inhibition/knockdown of Dnmt1 in other hemipterans (*O. fasciatus* Bewick et al., 2019; Cunningham et al., 2024; Washington et al., 2021 and *Bemisia tabaci* Shelby et al., 2023), and *dnmt1* is maternally expressed in the pea aphid (data not shown). However, distinct aspects of the observed phenotype may be *dnmt3*-specific. We observe defects in the germarium and in segregation of the oocyte after 5-azacytidine treatment that are not seen with other hemipteran species after *dnmt1* knockdown. Intriguingly, down-regulation of *dnmt1* in *B. tabaci* has been shown to cause a decrease in the expression of *cdc20* (Cunningham et al., 2024; Shelby et al., 2023), a gene involved in mitosis. It thus remains possible that *dnmt1* inhibition has an aphid-specific phenotype, but given its very broad and consistent effect in other species we think that unlikely. Differences in the function of DNA methyltransferases between the pea aphid and other hemipteran species could also reflect peculiarities of aphid oogenesis. Parthenogenetic aphids are able to activate their oocytes independently of fertilisation; haploid oocytes are arrested at prophase, and a modified meiosis II division gives rise to a diploid oocyte; aster self-organisation (Riparbelli et al., 2005) facilitates a single maturation division that gives rise to the diploid polar body (Blackman, 1978; Gautam et al., 1993; Le Trionnaire et al., 2008). Therefore, it is possible that the phenotypes we observe in the germarium, oocyte and follicle cell populations after 5-azacytidine treatment (Figures 6 and 7) are due to aphid-specific roles for the DNA methyltransferases in parthenogenetic oogenesis.

We also see defects in embryogenesis and patterning (Figure 7) as a result of 5-azacytidine treatment, with both premature and aberrant expression of *Ap-vasa* and *Ap-wg* (a marker of posterior cell fate and segmentation) as well as abnormal embryo morphology. These phenotypes may reflect defects in embryonic cell division similar to the defects that we see in oogenesis. Alternatively, in *N. vitripennis*, knockdown of *dnmt1* resulted in failure of cellularisation and arrest of embryogenesis at or around gastrulation that is consistent with dysregulation of the maternal-to-zygotic transition (Arsala et al., 2022; Zwier et al., 2012). It is therefore possible that the embryonic defects that we observe are due to similar dysregulation of the maternal-to-zygotic transition. We do also observe patterning defects that may be due to mis-localisation or mis-expression of maternal factors that are required to establish polarity of the embryo and the segmentation cascade (Lynch, 2019; Pechmann et al., 2021; Sachs et al., 2015). Consistent with 5-azacytidine treatment causing disruption of the embryonic axes, we do observe mis-expression of *Ap-vasa* at the anterior and dorsal surface of some embryos. Axis specification in viviparous

aphids may involve novel patterning molecules or mechanisms (Duncan, Leask, & Dearden, 2013), and it would be interesting to determine if 5-azacytidine treatment causes similar defects in oviparous reproduction, or if the role of DNA methyltransferases in axis specification is unique to viviparous reproduction in aphids. It has been suggested that germ cell specification relies on germ plasm preformed within the egg prior to cellularisation (Chang et al., 2006). Thus, it is possible that aberrant *Ap-vasa* expression is due to defects in this process, but this does not explain the mis-expression of *Ap-wg*. Importantly, we did not observe any defects in patterning or morphology in mid-embryogenesis; this may indicate that DNA methytransferases are only required for patterning or morphology in early viviparous development or may be indicative of resorption of severely affected oocytes and embryos (Ward & Dixon, 1982). In a related aphid species early embryos are resorbed, mid-stage embryos are paused and late-stage embryos continue development in response to starvation stress, which may indicate the existence of developmental checkpoints at these stages. Our data do indicate a lower reproductive rate between days 13 and 16, which may be consistent with embryo resorption (Figure 4).

Treatment with 5-azacytidine also resulted in the production of stillborn nymphs (Figure 5). This phenomenon was constrained to a relatively small temporal period during treatment, likely corresponding to a small window of development where embryos are sensitive. The appearance of stillborn nymphs over just three days is consistent with aphids younger than the critical period being resorbed or arrested (Ward & Dixon, 1982). What defects lead to the stillborn phenotype is unclear, but it could reflect a role for the DNA methyltransferases in processes critical for embryo maturation, such as rupture and withdrawal of the extraembryonic membranes (Horn et al., 2015; Schmidt-Ott & Kwan, 2016). However, the serosa of the parthenogenetic pea aphid is reduced compared with other hemipteran species (Miura et al., 2003) and the role of the extraembryonic membranes in viviparous aphid development is not yet clear. Nymphs older and younger than stillborn nymphs generally lived to adulthood and were able to reproduce at rates consistent with the offspring of control aphids (Figure S6). Rather than indicating that the DNA methyltransferases are not required specifically for maintenance of the germ line, this observation may reflect a period during development in which embryos are refractory to 5-azacytidine treatment or that embryos that were severely affected were resorbed or stillborn. Further studies using RNAi or CRISPR/Cas9 are required to fully define the roles of these two paralogs and *dnmt1* in pea aphid oogenesis and embryogenesis.

The co-expression of both *dnmt3* paralogs in the developing germ line raises the question of why both paralogs are maintained in the genome. It may be that *dnmt3x* has been released from the selective pressure towards its ancestral function and has acquired a new function through neo-functionalisation. Or, given that both are being expressed in the same way, it may represent a case of sub-functionalisation. While *Ap-dnmt3a* has a Cyt C5 DNA methylase domain (Figure S4) and is presumed to be functional as a DNA methyltransferase, *Ap-dnmt3x* has a PWWP motif essential for protein–protein interactions and interactions with chromatin (Kucharski et al., 2023) and an ADDz_Dnmt3 domain (Figure S4) but is lacking some of the conserved amino acid residues in the catalytic methylase domain that are thought to be required for activity as a DNA methyltransferase (Walsh et al., 2010). Therefore, Dnmt3x may function similarly to the vertebrate Dnmt3L (Walsh et al., 2010). Dnmt3L, while not able to actively methylate DNA itself, stimulates de novo methylation through methyltransferases that can (Chédin et al., 2002). As *Ap-dnmt3x* mRNA co-localises with *Ap-dnmt3a*, it is possible that it may play a similar role, interacting with and modulating the activity of *dnmt3a* in establishing methylation patterns. Interestingly, the unusual arrangement of dnmt3 homologues observed within the Aphidomorpha is reminiscent of the system described in *Daphnia magna*. *D. magna* also possess two *dnmt3* homologues, one of which possesses a putatively functional methyltransferase domain but is lacking a PWWP domain, while the other appears to have a diverged methyltransferase domain, presumed to be non-functional (Lindeman et al., 2019; Nguyen et al., 2020). Given that *D. magna* also exhibit cyclical parthenogenesis (Kleiven et al., 1992; Young, 1979), it is noteworthy that the unusual *dnmt3* configurations of aphids and *D. magna* are superficially consistent.

Cyclical parthenogenesis is thought to have evolved in Aphidomorpha over 220–150 MYA (Grimaldi & Engel, 2005), before the divergence of the phylloxerans and adelgids from the true aphids, while viviparity in the asexual phase arose in the true aphids, after the divergence (phylloxerans and adelgids reproduce exclusively via oviparous cyclical parthenogenesis) (Davis, 2012). As the duplication of Dnmt3 occurred in an ancestor of these three groups, it is tempting to speculate that one or both of these paralogs may have a role in mediating parthenogenesis. That the mRNA of both *Ap-dnmt3a* and *Ap-dnmt3x* is detected in the oocytes and germ cells of parthenogenetic viviparous pea aphids and that inhibition of the DNA methyltransferases causes defects in oogenesis and early embryogenesis is consistent with this hypothesis. Further research should focus on the role of DNA methyltransferases in oviparous reproduction in aphids, specifically investigating the effects of knockdown or knockout of dnmt3 paralogs on oogenesis and assessing their expression and localisation between morphs destined to be viviparous and oviparous. Similar functional studies in adelgids, phylloxera and scale insects (Coccomorpha) will be required to determine the ancestral roles of the DNA methyltransferases and will be essential to fully test this hypothesis.

AUTHOR CONTRIBUTIONS

Kane Yoon: Conceptualization; investigation; formal analysis; visualization; writing – original draft. **Stephanie Williams:** Investigation; writing – review and editing. **Elizabeth J. Duncan:** Conceptualization; funding acquisition; investigation; writing – original draft; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Kaplan–Meier survival curve of aphids following 5-azacytidine treatment. There was no significant difference in survival between 5-azacytidine treated aphids and DMSO controls (Cox proportional hazard, $p = 0.78$).

Figure S2. Conservation of genes involved in DNA methylation across Hemiptera and Thysanoptera. The phylogenetic relationships between species and divergence times (shown at nodes) are based on TimeTree of Life (Kumar et al., 2022). Lighter colours indicate the presence of a single homologue in the genome, while darker colours indicate the presence of paralogs (the number of paralogs is indicated. Dnmt1, Dnmt2 and Tet1) are generally very well conserved across the Hemiptera and Thysanoptera although duplicates of Dnmt1 are often seen, which may indicate the potential for neo or sub-functionalisation of these paralogs. In contrast, dnmt2 and Tet1 are never duplicated. Dnmt3 exhibits more variability, with independent duplications in Aphidomorpha, Thysanoptera, and a potential species-specific duplication in *Trialeurodes vaporariorum*, along with losses in the Coccoidea and multiple losses in the Heteroptera.

Figure S3. Schematic representation of synteny based on *A. pisum* dnmt3a and dnmt3x and flanking genes, with mapping of orthologues of *A. pisum* Dnmt3x and/or Dnmt3a encoding genes and the genes directly flanking them (identified using NCBI genome browser) from *B. tabaci*, a non-aphid hemipteran bug, and *M. persicae* (another aphid) to *A. pisum* chromosomes (using a chromosome-level *A. pisum* genome assembly). *B. tabaci* possesses only a single Dnmt3, more homologous to *A. pisum* Dnmt3a, and the genes flanking it do not appear on the *A. pisum* X chromosome (where dnmt3a and dnmt3x are located). For *M. persicae* flanking genes, three out of four appear on the X chromosome, nearby to dnmt3a and dnmt3x, but one appears on the A2 chromosome.

Figure S4. Domain structure of insect Dnmt3 proteins superimposed on species phylogeny. Domains identified by Batch CD-search at NCBI, and visualised using IBS 2.0 (Xie et al., 2022).

Figure S5. Conservation of Intron/Exon structure. (A) comparison of intron/exon structure between the pea aphid paralogs of dnmt3. (B) comparison of the intron/exon structure between three aphid species (*S. flava*, *M. persicae and A. pisum*) with representatives of the phylloxera (*D.vitifoliae*) and adelgids (*A. cooleyi*). Gene structures were generated using https://www.webscipio.org/search and then were compared using https://genepainter.motorprotein.de/genepainter.

Figure S6. Fecundity of nymphs produced by 5-azacytidine treated and DMSO-control mothers.

Table S1. Orthologs of DNA Methylation Machinery (Dnmt1, Dnmt3 and Tet1) Found in the Genomes and Transcriptomes of Selected Insects.

Table S2. Preparation of aphid artificial diet (Prosser & Douglas, 1992).

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